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The Klason Lignin Determination as Applied to Aspenwood with Special Reference to Acid-Soluble Lignin

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THE KLASON LIGNIN DETERMINATION AS APPLIED TO ASPENWOOD WITH SPECIAL REFERENCE TO ACID-SOLUBLE LIGNIN

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INTRODUCTION

More than a half century ago, Peter Klason (1) reported the use of strong sulfuric acid for the first time in the quantitative determination of lignin. Although the procedure has undergone numerous modifications since it was first proposed by Klason, it still remains essentially unchanged. Hydrolysis of pre-extracted wood with 72% sulfuric acid, followed by dilution and secondary hydrolysis under standardized conditions, is presumed to dissolve the carbohydrates and leave only the lignin as a filterable residue. Due to his pioneering effort in the development of the method, it has become known as "the Klason lignin determination," and lignin isolated in this way is synonymous with "Klason lignin."

The occurrence of difficulties in extending the Klason lignin determination from softwoods to hardwoods has been recognized for a long time. Freudenberg and Ploetz (2) recommended changing the sulfuric acid concentration to suit individual hardwood species. Although lignin was thought to be resistant to acid hydrolysis, Brauns (3) found that part of the "lignin" in beech and maple could be removed with 1% sulfuric acid, and he stated that standard lignin determinations were not suitable for deciduous woods. Nevertheless, the Klason lignin determination enjoys great popularity in its application to industrial hardwood species.

The use of these generally cheaper and more abundant species has been increasing gradually for some time, and they now supply a substantial portion of the papermaking fiber used by the industry. This has

been promoted through the ability to produce competitive commercial pulps from hardwood by the neutral sulfite semichemical (NSSC) pulping process, and the shortage of spruce has led many sulfite mills in the Lake States to use the two indigenous aspens, Populus tremuloides and Populus grandidentata (4).

A suitable lignin analysis is becoming increasingly important with the expanding use of these woods. Although some feel that errors encountered in applying the Klason procedure to aspenwood in particular are rather insignificant, Pearl (5) recently questioned the status of the aspenwood Klason lignin when he found it contained only 65% of the methoxyl in the wood. Because it is unlikely that more than 10-20% of the total methoxyl is associated with carbohydrate, a significant amount of lignin may be "solubilized" during the Klason procedure.

An adequate critical evaluation of hardwood processes, both by the research chemist and by the producer, appears to be restricted at the present time because of the lack of a satisfactory method for the determination of lignin in these woods. This thesis is submitted as a fundamental contribution to this problem. The present investigation emphasizes the fate of aspen lignin during the standard Klason procedure and permits a comparison of the action of sulfuric acid on the lignin in wood and in other lignin preparations including Björkman's (6) milled wood lignin. Differences are evaluated in the light of the possible effects of sulfuric acid on lignin, and conclusions are drawn regarding the validity of the Klason lignin determination for the particular species (Populus tremuloides) of aspenwood investigated.

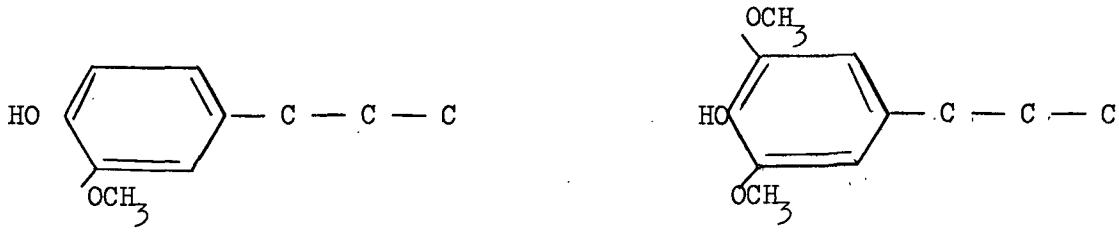
BACKGROUND DISCUSSION

Many aspects of the broad spectrum of lignin chemistry are important to this investigation, but their diversified nature makes it impracticable to incorporate them with the necessary detail in an integrated discussion of the determination of lignin. For this reason the following discussion is presented in separate chapters, with no real continuity between the chapters, other than the importance of each to the development and understanding of the experimental program.

THE NATURE AND DEFINITION OF LIGNIN

Current views on the structure of lignin are fragmentary and incomplete, and at the present time our understanding of the protolignin system is largely based on analogy and speculation (7). In spite of our hazy picture of lignin, certain features stand out rather clearly. Early spectrochemical studies such as those of Herzog and Hillmer (8, 9) supported the view that isolated lignin has a benzenoid structure, and the aromatic nature of lignin *in situ* was demonstrated by Lange (10). Its hypothesized formation from phenylpropane building stones was substantiated by Harris and co-workers (11) when a large portion of aspen methanol lignin was isolated as propylcyclohexane derivatives after high-pressure hydrogenolysis. The oxidation and ethanolysis studies of Hibbert and co-workers (12, 13) have demonstrated the construction of softwood lignins chiefly from guaiacylpropane monomers, and hardwood lignins from both guaiacylpropane and the corresponding syringylpropane building units. The early concept of lignin as a phenylpropane polymer has attained the status of a well-founded theory, and as discussed by

Adler (14), attention is now being directed toward the detailed structure, the nonaromatic portions of the molecule, and the ways in which the methylated phenylpropane units are joined together. It is here, however, that our picture fades quickly into the realm of analogy and speculation.



Guaiacylpropane Syringylpropane

Figure 1. Structural Units of Lignin

The presence of methoxyl groups in lignin serves as one of its most important analytical criteria. From studies on the stability of methoxyl in lignin and model compounds, Freudenberg and co-workers (15) concluded that the methoxyl group must be attached to the aromatic ring as a phenolic ether. Isolated softwood lignin generally contains about 15-16% methoxyl (16), and the higher (20.5-21.5%) value for hardwood lignin is consistent with the presence of the syringylpropane building unit.

Unfortunately, this characteristic offers little help in evaluating methods for the quantitative determination of lignin in wood. The average methoxyl content of aspen protolignin is unknown, and even the percentage of the total methoxyl associated with the lignin is open to question. Jones and Wise (17) identified 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-α-D-xylose produced on hydrolysis of aspen sawdust,

but the presence of methylated carbohydrates in hardwoods corresponding to more than 1-2% of the total methoxyl has not been demonstrated. The isolation and fractionation of aspen holocellulose by Thomas (18) indicated that about 10% of the methoxyl in aspenwood may be associated with carbohydrates, but this must be considered only a tentative value owing to the uncertainty in determining the lignin content of these materials.

The definition of lignin is a difficult task; not only is the exact structure still unknown, but the term "lignin" does not designate a constitutionally defined compound. Brauns (19) has approached the problem by defining lignin as being made up mainly, if not entirely, of phenylpropane building stones with the qualifications that it is unhydrolyzable by acids, easily oxidizable, soluble in hot alkali and bisulfite, and condenses readily with phenols and thio compounds. The "unhydrolyzable" restriction assumes that there are no groups such as acetyl or glucosidic carbohydrate which are removed by hydrolysis. Brauns (19) further states that it carries the major part of the methoxyl content of the plant, and that the methoxyl content is the main indication of its purity.

The concept of "lignin" was discussed by Kratzl and Billek (20) who also feel that a single lignin criterion (e.g., the Klason lignin determination or the nitrobenzene oxidation) is insufficient to define a substance beyond doubt as lignin, and they list a series of applied reactions which may be used to test lignin preparations. However, it was found that such criteria served to distinguish lignin from other wood components, without clearly defining the lignin itself. Wacek and

Schroth (21) use as their criteria for membership in the family of lignin, construction from guaiacyl or syringyl propane building units, and this is implied by Campbell and McDonald (22) when they state that the only satisfactory definition of "lignin" is the nonpolysaccharide portion of the wood cell wall in an extractive-free wood.

THE ISOLATION OF LIGNIN

The hope has been to evolve a method for the separation of the protolignin system without alteration from the other wood constituents, but this has never been achieved. This has been the major difficulty in the chemistry of lignin, and particularly in work directed toward the determination of its structure (23). Because an isolation procedure in which there is little physical or chemical change would yield a preparation most closely resembling protolignin, there has been much interest in dissolving lignin directly from the wood in solvents which will not react with it. The wood has been pretreated also in various ways in order to make the lignin more "accessible" to these unreactive or neutral solvents.

Only a small portion of the lignin can be isolated from wood meal with neutral solvents, and this has been described by Brauns (24) for sprucewood and later by Buchanan, et al. (25) for aspenwood. Freshly ground wood meal pre-extracted with cold water and ether is exhaustively extracted at room temperature with alcohol. The nonlignin components in the alcohol extract are removed from the lignin on the basis of their water and ether solubility, and the lignin obtained as a light cream-colored powder is generally referred to as "Brauns' native lignin" (BNL).

Its similarity in the case of spruce to the rest of the protolignin has been the basis for its selection as a lignin standard by many investigators in the past.

It would be incorrect, however, to regard BNL as identical with the rest of the protolignin. Spruce BNL and its sulfonic acids contain more phenolic hydroxyl than spruce lignin and lignosulfonates (26), and it almost certainly has a lower molecular weight (27). Freudenberg (28, 29) claims that 30-40% of BNL from spruce is a difficultly crystallizable lignan, but Brauns would not consider that portion of the total wood extract which is soluble in benzene or ether part of the native lignin. Kudzin and Nord (30) suggest that hardwood native lignin serves as a transient substrate phase which is converted enzymatically into residual (not extractable with alcohol) lignin with a higher methoxyl content.

The suitability of aspen (Populus tremula) BNL as a standard for aspen protolignin must be doubted in the light of Smith's (31) observation that p-hydroxybenzoic acid accounts for about 10% of its weight. Stanek (32) concluded that some of the p-hydroxybenzoic acid in aspenwood (Populus tremuloides) is combined in the cell wall, and possibly with the lignin. Nakano and co-workers (33) found more than 0.9% p-hydroxybenzoic acid in extractive-free aspenwood (Populus maximowczii), and it was assumed to exist as an ester group in the lignin. Although there is reason to believe that some p-hydroxybenzoic acid is associated with aspenwood lignin, it appears unlikely to represent anything close to 10% of the total lignin.

Björkman (27, 34-37) has demonstrated that up to 50% of the protolignin can be rendered soluble in aqueous dioxane if wood meal is ground in a vibratory ball mill. The milled wood lignin (MWL) extracted from this finely ground wood always contains a small amount of carbohydrate which appears to be chemically combined with the lignin. Björkman (34) concluded that MWL is more closely related to the bulk of the lignin in wood than Brauns' native lignin, and Adler (14) also believes that MWL is reasonably close to protolignin. According to Gierer (38) the isolation of lignin in a chemically unaltered state has not been achieved yet, but MWL in his opinion is a close approximation to protolignin. Grohn and co-workers (39), however, suggest there may be oxidative demethylation of the lignin during milling, and the question arises, of course, whether or not the chemical nature of lignin is changed in the ball mill.

The action of the vibratory ball mill and the sensitivity of materials to mechanical degradation during milling were discussed by Björkman (37). There are two outstanding features of the degradation:

(1) The degradation is based on a specific mechanical excitation process which appears to be essentially independent of secondary influences such as oxidation or thermal decomposition.

(2) Mechanical depolymerization in the vibratory ball mill is apparently limited to higher molecular weight materials as evidenced by the concept of a limiting D.P.

From studies on the vibration grinding of cellulose and polystyrene, Hess, Steurer, and Fromm (40, 41) attributed the grinding action to a localized mechanical excitation process distinct from thermal or oxidative degradation. Although the temperature increase for points of collision

may be as high as 46°C. (37), the grinding action between 0 and 90°C. was affected very little by temperature changes in contrast to the large temperature coefficient for both oxidation and thermal decomposition reactions. It was inferred (40, 41) that the mechanism is based on an independent activation and splitting by the grinding action proper, and localized compressive stresses over the surface area occupied by a C—O bond, for example, are thought to reach the required separation stress. The distinction between this "mechanicochemical" process and thermal degradation would lie in the distribution of energy. In the first case there would be a concentration of vibrating energy only at specific locations in the molecule, whereas in thermal degradation there would be an energy distribution over the entire molecule.

The concept of a limiting D.P. in the vibratory grinding of polymeric materials evolved from the observation that the rupture of primary valence bonds was generally limited to substances of higher molecular weight. Steurer and Hess (42) found that sucrose could not be degraded and showed by graphical extrapolation that a D.P. of 27 would be reached eventually when cellulose was milled. A simple statement of their explanation for this might be that as milling decreases the size of the molecules, the total effect of secondary valence forces decreases, and the movement of the molecules is no longer restricted to the point that the breaking of primary valence bonds is possible. Grohn and Augustat (43) have shown also, however, that the limiting D.P. for a particular material can vary with the grinding conditions. Our understanding of the action of the vibratory ball mill is admittedly incomplete, but the concept of a limiting D.P. has been well established.

The limiting D.P. reported by Björkman (34) for MWL isolated under specific grinding conditions corresponded to a molecular weight of 11,000, or about 60 phenylpropane units. End-group effects should be minimized with this relatively large D.P., and the nature of the degradation suggests that lignin within this "unit" may remain essentially identical to protolignin. A note of caution is introduced, however, by the implications of oxidative demethylation (39). Although it is still too early to draw definite conclusions about the chemical identity or possible differences between MWL and protolignin, MWL appears to be the most satisfactory "standard" now available to the lignin chemist.

THE DETERMINATION OF LIGNIN

There is no completely satisfactory method now available for the quantitative determination of lignin in wood. Unless a procedure is found that permits the isolation of lignin in an unchanged pure state, no undisputed quantitative method can be developed (44). The isolation of such a "lignin" may be untenable if there are chemical bonds between lignin and carbohydrate, and the feasibility of developing an undisputed method may be open to question.

The methods for the direct determination of lignin in wood have always rested on somewhat arbitrary procedures, and those commonly used, such as the Klason sulfuric acid method, are based on hydrolysis of the polysaccharides by strong mineral acids. There is a tendency for pectins, proteins, resins, and other extractives to become insoluble during the acid treatment, and sulfuric acid itself may be occluded. Other factors which may increase the "apparent" Klason lignin are incomplete hydrolysis

and removal of the carbohydrate, or humification and condensation of carbohydrate degradation products with the lignin. Part of the "true lignin" may be solubilized by the acid, and this would include water which is split off during condensation of the lignin. Any acid-soluble lignin appearing in the Klason filtrate would not be weighed as Klason lignin, and there would be a corresponding drop in the "apparent" lignin content of the wood.

The interference produced in the Klason lignin determination by extraneous components and extractives has been discussed by Browning (45) and Brauns (46), and the observed lignin values in the case of mature aspenwood should not be affected significantly by these materials. Pectin and protein, for example, occur only to a small extent in mature wood (46), and resins, waxes, and other extractives can be removed generally with organic solvents so that their effect is minimized.

Marth (47) found only 0.002% sulfur in aspenwood, but the sulfur content of Klason lignins prepared from this wood was extremely variable over the range of 0.20 to 0.64%. With more thorough washing procedures it could be reduced to about 0.10%, and the presence of the sulfur was attributed mainly to sorption of sulfuric acid by the lignin.

There are many factors which affect the hydrolysis and removal of carbohydrate during the Klason lignin determination. The time, temperature, and acid concentration are all important and interdependent variables. The selection of an optimum acid concentration, however, may depend upon the species of wood under investigation as well as the time and temperature of the initial hydrolysis.

Softwood lignin is understood to be characterized by insolubility in sulfuric acid of all concentrations, and its determination presumes the removal of certain impurities. At sulfuric acid concentrations below 60 to 65%, some polysaccharides remain in the lignin; this is the lowest acid concentration at which cellulose can be dissolved. At acid concentrations which are too high humic substances appear, and the "lignin" begins to increase as shown in Fig. 2 for sprucewood (48).

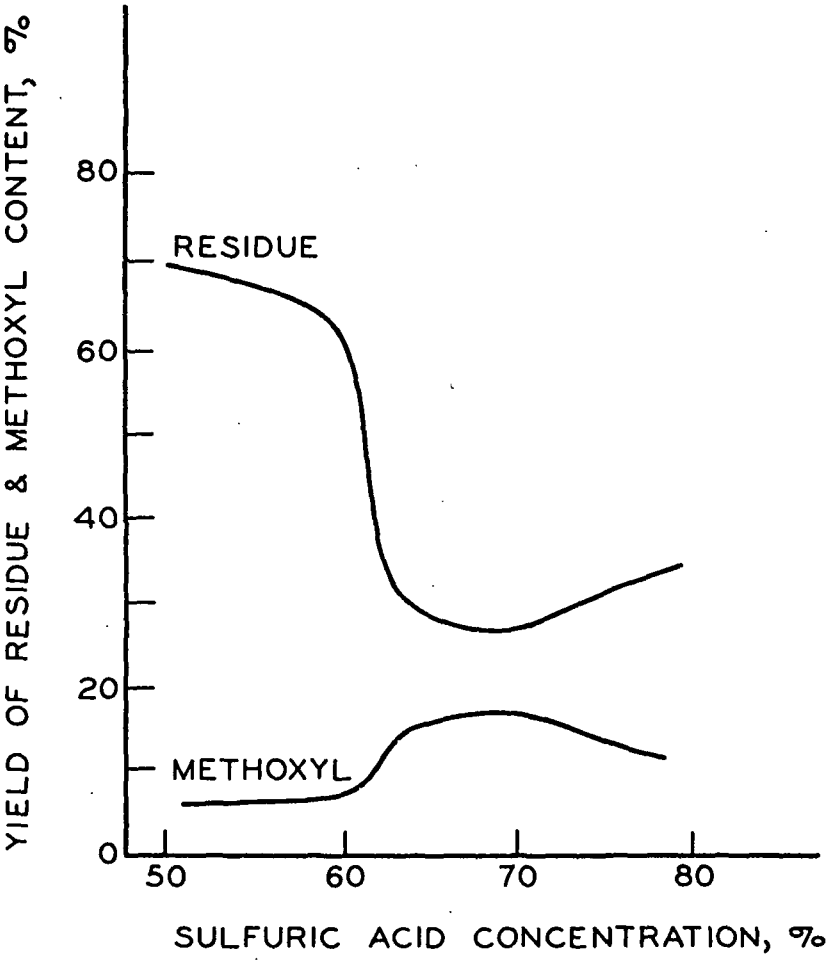


Figure 2. Isolation of Spruce Lignin with Sulfuric Acid (16 Hr. at 10°C.)

Selection of the acid concentration giving the minimum lignin value, in order to minimize interference from nonlignin impurities, is often justified by the existence of a corresponding maximum for the methoxyl content of the isolated lignin.

Freudenberg and Ploetz (2) reported that the lignin determination may fail completely with certain hardwoods when no minimum lignin value is found. The methoxyl content passes through a maximum in all cases, however, and the quantitative determination can be carried out with that acid concentration giving a lignin with the highest methoxyl content. The wood residue isolated in this way was called "sulfuric acid lignin" to distinguish it from the ordinary Klason lignin.

The difficulty observed in hardwood lignin determinations motivated the study of the Klason procedure reported by Thomas (49), but no unusual effect was encountered in the case of aspenwood. The acid concentration giving the minimum lignin value produced a lignin with the highest methoxyl content, and it was concluded that in the analysis of aspenwood, 72% is the most suitable concentration of sulfuric acid for the lignin determination.

A 72% sulfuric acid concentration has proved a satisfactory compromise for the lignin determination on most pulpwood species, and the factors affecting its use have been studied thoroughly by Ritter, Seborg, and Mitchell (50). In general, for a given acid concentration, if the temperature is raised to increase the reaction rate, the initial hydrolysis period can be decreased. After the initial hydrolysis, the mixture must be diluted and boiled to hydrolyze and render soluble all the

polysaccharides and coagulate the finely divided insoluble ligneous material so that the filtering and washing of the residue may be facilitated. The recommended procedure (50) calls for an initial two-hour hydrolysis at 20°C., dilution of the sulfuric acid to 3%, and boiling the mixture four hours to complete the secondary hydrolysis. The initial hydrolysis period may be extended to three hours to assure complete removal of the carbohydrate with no significant effect on the lignin values as a result of increased humification of the carbohydrate.

The interference caused by humification and condensation of carbohydrate during the Klason lignin determination was investigated by Norman (51). Pentosans, and particularly xylans, were found to produce furfural in the presence of 72% sulfuric acid. It was suggested that the cause of the disturbance is the condensation of this aldehyde with lignin and not necessarily the formation of an insoluble condensation product from furfural itself. Xylose was added to various lignified materials prior to a Klason lignin determination, and the increase in "lignin" was not large in comparison to the high proportion of pentose added. Norman (51) stated that although pentose-containing polysaccharides of lignified materials may not be causing any serious errors in the lignin determination, it cannot be said they are wholly without effect.

It has been recognized for a long time that sulfuric acid is not without an effect on lignin--even on softwood lignin. According to Brauns (46), native spruce lignin lost about 8% by weight on treatment with 72% sulfuric acid, and the carbon analysis increased from 63.7 to

65.4%. The methoxyl content was increased also from 14.8 to 15.5%, a value often found for Klason lignin from sprucewood, and some material low in methoxyl must have been removed. Water may have split off during condensation of the lignin, and another portion of the lignin could have been solubilized as well.

The loss of water through condensation of lignin in the presence of sulfuric acid was studied by Müller and Dobberstein (52). The hydrolysis of beechwood with 74.9% sulfuric acid at 20°C. was followed by continuous conductivity measurements, and it was concluded that about 13% of the protolignin was eliminated as water during the hydrolysis. Undoubtedly, some water is lost in this way, but changes in the analytical composition of isolated lignins, when subjected to the standard lignin determination, indicate that this effect could not normally be of the magnitude suggested by Müller and Dobberstein (52).

The solubilization of softwood lignin during the Klason lignin determination has been studied by several investigators with rather contradictory results. Brauns (46), of course, found that 8% of native spruce lignin was lost, but the relationship between BNL and protolignin is still open to question. Campbell and McDonald (53) and Wacek and Schroth (54) claimed that soluble lignin from spruce may amount to almost 6% of the wood, or approximately 20% of the lignin. This is disputed by Richtzenhain and Dryselius (55) who concluded that the Klason filtrate contained only very small amounts of lignin. Loras and Løschbrandt (56) found that the lignin lost in the Klason filtrate from sprucewood was of no importance in the gravimetric determination

of lignin. The detection of only 0.3% acid-soluble lignin from sprucewood by Browning and Bublitz (57) also supports the hypothesis that softwood lignins are essentially unhydrolyzable by acid.

Perhaps the results regarding the formation of acid-soluble lignin are even more contradictory in the case of hardwoods. Campbell and McDonald (53) estimated that acid-soluble lignin from beech may be as high as 7.4% of the wood, but this was not upheld by Richtzenhain and Dryselius (55). The latter investigators contended that the Klason filtrates contain little lignin, and based on the weight of the wood, it amounts to less than 1% and 1.3% for aspen and beech, respectively. The possible formation of rather large amounts of soluble lignin was confirmed by Stewart and co-workers (58), however, when they found almost 4% soluble lignin in the Klason filtrate from E. regnans.

In a recent study of aspen lignin, Pearl and Beyer (5) reported a methoxyl balance for the Klason lignin determination on aspenwood. The recovery amounted to essentially 100%, and only a small amount of methoxyl (7.5%) split off during the procedure. It showed, however, that only 65% of the methoxyl was found in the Klason lignin, and it was hypothesized that a large portion of the true lignin was solubilized during the standard lignin determination.

In a discussion of this hypothesis, Pew (59) stated that his data did not bear out the suggestion of Pearl that the TAPPI lignin content may be considerably lower than the true value. One argument is based upon the observation that the TAPPI lignin is in agreement with the yield of enzyme lignin after correction for residual carbohydrate.

The enzymatic degradation was carried out at pH 4.6 and 40°C. for several days, and part of the lignin may have been solubilized. Furthermore, the relationship between enzyme lignin and protolignin may be somewhat doubtful (60). The arguments based on ultraviolet absorption studies were confusing, and the data actually support the contention that Klason lignin values are lower than they should be.

THE NATURE AND MEASUREMENT OF ACID-SOLUBLE LIGNIN

With even the existence of acid-soluble lignin still a matter of debate, it is not surprising that its exact nature remains in doubt. Wacek and Schroth (54) believe that protolignin is composed of two separate fractions, protolignin I and protolignin II. Protolignin I is not precipitated as acid lignin under usual conditions, and it is most likely combined with carbohydrate since it is most resistant to oxidative attack. Stewart, et al. (58) claim that soluble lignin is derived from the lignin in situ, and that it does not pre-exist as such in the wood. It was suggested that soluble lignin may consist of low molecular weight degradation products which are formed during the 72% sulfuric acid treatment, and not during subsequent boiling with 3% sulfuric acid.

In applying the Klason lignin determination to wood, it has been observed for many years that additional material similar to the Klason lignin may precipitate from the acid filtrate upon standing (57). Although this fraction of the acid-soluble lignin may be isolated easily, a quantitative isolation is plagued with difficulties. Chief among them has been the inability to evaluate the isolation procedures. Unless a way is found to do this, no satisfactory method is apt to be developed.

Two unique approaches to the isolation of soluble lignin have been reported in the literature. The first was the basis for a quantitative procedure reported by Migita and Kawamura (61) in which the concentrated Klason filtrate was extracted with butanol to remove the lignin. The second was that of Campbell and McDonald (53) who passed the Klason filtrate through a column of "Zeo-Karb 215" cation-exchange resin, and isolated the adsorbed lignin by eluting the column with alcohol. This procedure was utilized later for a gravimetric determination by McKenzie, McPherson, and Stewart (58) who found it necessary to make a correction for the alcohol blanks from the column. In attempting to use this method, Marth (47) found the blanks extremely large and variable, but this may have been due to the critical effect of temperature on the alcohol solubility of the resin (62).

Stewart (63) described an extrapolation procedure which served to estimate both the total lignin content of wood and the amount of acid-soluble lignin in the Klason filtrate. An isolated Klason lignin from E. regnans was given successive treatments according to the Klason procedure. A loss in recovered material occurred at each step, and after three additional treatments, only 77% of the original Klason lignin was recovered. It was assumed that lignin was lost in isolating the original Klason lignin from the wood as well, and an extrapolation back to "0" treatments gave an estimated value for the protolignin. This was approximately 27% in comparison to 22.3% for the original Klason lignin, and the difference between these values indicates that as much as 15-20% of the protolignin may have been solubilized during the Klason lignin determination on the wood.

Ultraviolet spectrophotometry has been the one method most used and investigated in the past for the determination of acid-soluble lignin. The measurement itself is based upon the fact that strong molecular absorption in the ultraviolet is usually associated with structures capable of resonance between several configurations such as a system of conjugated double bonds (64). The benzene nucleus, for example, absorbs in two regions of the accessible ultraviolet; there is a low intensity band about 240-260 mμ and an intense band around 200 mμ (65). Substitution in the ring, particularly of oxygen, has a pronounced effect on the position and intensity of the two maxima (64). Inasmuch as the lignin molecule contains no large portion of unsaturated aliphatic units in addition to its aromatic structure, Jones (64) concluded that the two characteristic bands in the lignin spectrum at 200-230 and 275-285 mμ are specifically attributable to the oxygen-substituted benzene nucleus in lignin. This is the basis for the determination of acid-soluble lignin from the ultraviolet absorption characteristics of the Klason filtrate.

There are two major difficulties, according to Browning (57), which limit the usefulness of ultraviolet spectrophotometry as a promising technique for the measurement of acid-soluble lignin. The first is the uncertainty imposed by the presence of furan-type compounds in the Klason filtrate, and the second is the questionable nature of any value assigned to the absorptivity of the material being studied. Both of these difficulties must be overcome if ultraviolet spectrophotometry is to afford a reliable means of estimating soluble lignin in the Klason filtrate.

The necessity for caution in ascribing the ultraviolet absorption maximum at 280 mμ in the Klason filtrate to the presence of lignin was pointed out by Richtzenhain and Dryselius (55). Glucose and xylose treated with 72% sulfuric acid and diluted to an acid content of 3% quickly developed absorption spectra resembling those of lignin (max. ca. 280 mμ) upon heating, and the absorbance continued to increase with additional heating. This was confirmed by Love (66) who found that the spectra of sugars after heating in sulfuric acid are caused by varying amounts of at least four different substances which absorb strongly in the region of 255-315 mμ. The spectra of xylose and glucose closely resembled furfural and hydroxymethylfurfural, respectively. Marth (47) found that a Klason filtrate which was boiled with reflux rather than without had a much stronger absorbance at 280 mμ, and this was attributed to the presence of additional furfural which could not be steam distilled under reflux. There is no doubt that furan-type compounds confound acid-soluble lignin determinations which are based on absorption measurements in the near ultraviolet.

Attempts have been made to overcome this deficiency in the ultraviolet absorption technique for the determination of soluble lignin. Løschbrandt (67) extrapolated the hydrolysis time curve to zero time in order to circumvent the carbohydrate absorbance. Loras and Løschbrandt (56) dialyzed the Klason filtrate to remove the relatively small molecules of furfural and hydroxymethylfurfural. It was argued that although part of the lignin may pass the membrane, even a large number of small lignin fragments would count comparatively little as a weight fraction. Browning and Bublitz (57) calculated the lignin concentration

from absorbance measurements at 280 and 215 mμ and assigned absorptivities to the carbohydrate degradation products in order to do so. These methods are not without limitations, and a satisfactory solution to this problem remains to be found.

This emphasizes the importance of recent publications by Kleinert and Joyce (68, 69) reporting studies on the absorption characteristics of lignosulfonates at 205 and 280 mμ. Most interesting is the conclusion that absorption at 205 mμ is not influenced by furan-type compounds and exhibits a close relationship to lignin substances in spent liquors. Absorption measurements at this wavelength were apparently successful in following the progress of delignification during the course of a bisulfite cook with a commercial mill digester. This was confirmed by Schöning and Johansson (70) whose study of spent sulfite liquor showed that the absorption at 205 mμ was due only to lignin.

Kleinert and co-workers (71, 72) continued to investigate the feasibility of measuring different types of lignin by means of light absorption in the far ultraviolet. This technique was applied successfully to kraft cooking liquors, brown stock washers, and alkali-soluble lignin from pulps using alkali lignin as a reference standard. More recently Marraccini and Kleinert (73) found that the 205 mμ absorption maximum could be used as an empirical tool for estimating lignin in acid hydrolyzates of unbleached, low-lignin pulps. Its successful application to Klason filtrates from wood could overcome a major obstacle in the quantitative determination of acid-soluble lignin, the uncertainty imposed by the presence of carbohydrate degradation products in the filtrate.

A serious limitation in the spectrophotometric determination of lignin arises from the necessity to know the relationship between light absorption and lignin concentration. Thus, the unavoidable selection of a reference standard, particularly in cases where the nature of the lignin is open to question, often limits the usefulness of this procedure. These considerations, for example, led Bjorkqvist, et al. (74) to state that spectrophotometric methods cannot be used to measure lignins in pulps because different lignins are measured in different pulps.

This limitation may be cause for even greater concern in the determination of soluble lignin in the Klason filtrate from wood. Brauns native lignin from the species in question is generally selected as the reference standard, but not without reservation. It is known that isolation procedures yielding dark lignin preparations can cause significant changes in the absorption characteristics of lignin (75), and acid-soluble lignin may be quite unlike protolignin and BNL in this respect. More will have to be learned about the nature and absorption characteristics of this material before it can be determined spectrophotometrically with confidence.

PRESENTATION OF THE PROBLEM

STATEMENT OF THE PROBLEM

Inasmuch as confusion exists in the literature over the validity of the Klason lignin determination, this thesis was undertaken to study the fate of aspenwood lignin during the Klason lignin determination, and to evaluate contradictory reports about the status of the aspenwood Klason lignin. More specifically, it was the purpose of this work to study the formation of acid-soluble lignin and attempt to answer the following questions:

(1) Is any significant amount of lignin solubilized during the Klason lignin determination?

(2) Can acid-soluble lignin be measured quantitatively?

(3) What is the nature of this material and its relationship to the Klason lignin itself?

APPROACH TO THE PROBLEM

Ultraviolet spectrophotometry utilizing the lignin absorption maximum at 205 mμ offered the best hope of overcoming carbohydrate interference and establishing the presence of acid-soluble lignin in the aspenwood Klason filtrate. Accordingly, absorption characteristics of acid hydrolyzates from the wood and various sugars were studied with a DK-2 recording spectrophotometer. Conclusions were drawn about the existence and formation of acid-soluble lignin, and the feasibility of obtaining absorbance values characteristic only of the lignin itself.

Acid-soluble lignin might be determined quantitatively as well through the use of ultraviolet spectrophotometry if its absorption characteristics could be ascertained with reasonable certainty, and aspen milled wood lignin (MWL) was isolated as part of a program which endeavored to solve this problem. A vibratory ball mill was constructed which was similar in principle to the one used by Björkman (34), but a special cooling chamber was incorporated in the design which allowed sub-zero grinding temperatures to be maintained. Thus, in an attempt to promote the grinding action and minimize any thermal side effects, the temperature was kept about -78°C. while the wood was milled. MWL was extracted from this finely pulverized wood with aqueous dioxane and purified according to the "standard method" of Björkman (34).

The MWL was characterized and subjected to the Klason lignin determination in order to study the solubilization of lignin and select a reference standard for the spectrophotometric determination of acid-soluble lignin. The ultraviolet absorption characteristics of MWL and other lignin preparations were compared with those of the Klason filtrates from wood and from MWL. This permitted conclusions about the reliability of MWL as a standard for acid-soluble lignin, the amount of lignin in the Klason filtrate, and the relationship between MWL and protolignin.

Various methoxyl balances and chromatographic investigations were included in further attempts to learn more about the nature of acid-soluble lignin and its relationship to the Klason lignin.

EXPERIMENTAL PROCEDURES, RESULTS AND CONCLUSIONS

PREPARATION AND CHARACTERIZATION OF THE STARTING MATERIAL

WOOD PREPARATION

Three aspen (Populus tremuloides) trees 29-35 years old and 50-65 feet in height were felled at the Rhinelander Paper Co. Experimental Forest, Eagle River, Wisconsin, between May 26 and May 28, 1958. Bolts were obtained from each tree, barked, and scraped to remove soft xylem. The bolts were split, knots drilled out, and any dark sections or decay removed by hand. The sound wood was then reduced to sawdust on a circular saw and air dried. The airdry material from each bolt was ground separately in a Wiley mill to pass a 28-mesh screen; only a small fraction had to be reground, and overheating was easily avoided with sufficiently dry wood. The moisture contents of the samples were determined and a composite 3000 g. (o.d. basis) sample was made up consisting of 1000 g. from each bolt.

A 640-g. portion (598 g. o.d.) of the composite sample was extracted with 95% ethanol:benzene (1:2) in a large Soxhlet extractor for 36 hr., washed with diethyl ether and cold water to remove any alcohol or benzene, and allowed to air dry. The alcohol-benzene extract and washings were combined and evaporated to dryness, and methoxyl determinations were made on the wood and extractives according to Institute method 18. The methoxyl balance is shown in Table I.

TABLE I

METHOXYL BALANCE FOR EXTRACTION OF ASPENWOOD

Basis: 100 g. ovendry wood meal with 5.41 g. methoxyl

Extractives	3.6	
Methoxyl in extractives		0.43
Extracted wood (by difference)	96.4	
Methoxyl in extracted wood		5.04
	100.0 g.	5.47 g.

PREPARATION OF MILLED WOOD LIGNIN

The Vibratory Ball Mill

The vibratory ball mill developed by Forziati, et al. (76) at the National Bureau of Standards was used by Björkman (34) in the preparation of MWL, and a similar mill was constructed for this investigation. The drawings and specifications of the N. B. S. mill were obtained through the courtesy of F. H. Forziati[1], and the basic design was modified to provide a cooling chamber around the grinding vessel. Details of the mill can be found in APPENDIX I and elsewhere (34, 76), and only a brief description is presented here.

The mill consists of a horizontal shaft connected at one end, by means of reinforced rubber tubing, to a 1/4-horsepower electric motor operating at 1,800 r.p.m., and at the other end to an eccentric weight which rotates in a housing suspended from leaf springs. A

[1] Chemist, Textiles Section, N. B. S., Washington 25, D. C.

cylindrical jar of approximately 1-liter capacity, surrounded by a concentric cooling chamber of the same capacity, fits into a holder which is fastened to a slip ring. The slip ring and holder are bolted together, but in such a way that they remain insulated from one another. The slip ring is held by the housing so that operation of the mill causes it to rotate (ca. 1 r.p.m.) automatically with the jar assembly[1] about an axis coincident with the centerline of the horizontal shaft. This counteracts any tendency for the wood to settle in the jar and promotes more uniform grinding. The vibratory movement of the jar assembly and housing is brought about by the rotation of the eccentric weight. This sets steel balls within the jar into rapid motion, and their collisions grind wood sawdust placed in the jar with them. When grinding at reduced temperatures, the jar assembly is insulated and a refrigerant added periodically to the cooling chamber.

The jar and cover were machined from 440-F stainless steel, a heat-treatable material which can be hardened to Rockwell "C" 59, and 1/4-inch ball-bearing balls of the same material and hardness were commercially available. Björkman (34) found that chromium-plated tool steel was easily damaged and subject to corrosion, and that the softer stainless steel was easily abraded, causing an objectionable contamination of the wood. The use of 440-F stainless steel minimizes corrosion and contamination which may be associated with the grinding operation.

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[1] Jar, cooling chamber, and holder.

Preliminary Studies with the Vibratory Ball Mill

The Effect of the Ball Mill on α-Conidendrin

Although degradation in the vibratory ball mill is generally believed to be restricted to materials with a high molecular weight, Browning (77) observed that a "Wig-L-Bug" used to prepare infrared samples can change the infrared curve of compounds such as conidendrin. It was suspected that the ball mill, which may be quite similar in action to the "Wig-L-Bug", could have a similar effect on these compounds. If this were indicative of the chemical alteration of simple compounds, there would be serious grounds for objecting to the use of MWL as a standard for protolignin.

α-Conidendrin (m.p. 254-256°C.) was milled 4 hr. at room temperature in the vibratory ball mill. The milled and unmilled samples were hand ground with KBr and infrared absorption curves determined in the solid phase. Some new bands were formed and peaks shifted as a result of milling, but there was no change in the exceptionally high melting point. Nevertheless, the changes in the infrared curve were significant enough to require further proof that some chemical change had not occurred.

White and co-workers (78) demonstrated recently that crystallinity affected the infrared spectrum of sucrose, and it seemed possible that the changes in the infrared spectrum of the milled sample also reflected a crystallinity effect. It was reasoned that if this were the explanation for the difference in the samples, the effect would disappear in solution. This was borne out by finding that the infrared

curves of the two samples in chloroform were identical in the 2-12 μ range. In addition, the milled sample in the solid phase was like either sample in solution, and it was concluded that there were no detectable chemical changes produced on milling. This supports the theory that the degradation is limited to materials with a high molecular weight.

Isolation procedures for MWL

The time and temperature were the principal variables studied in preliminary work designed to select suitable grinding conditions and develop techniques for the preparation of MWL. It was hypothesized (40-42) that mechanical depolymerization in the vibratory ball mill is essentially independent of temperature. If the release of lignin from wood depends upon depolymerization of the lignin molecule, or rupture of true chemical bonds (lignin-carbohydrate or carbohydrate-carbohydrate) holding the lignin, the temperature should have no effect on the grinding efficiency of the mill. Normal grinding reactions, concerned only with a decrease in particle size, would be expected to have an improved efficiency through temperature reduction which makes the material more brittle. Thus, if the release of MWL depends upon physical phenomena and hydrogen bonding as suggested by Björkman (34), grinding at sub-zero temperatures might not only minimize thermal side effects, but promote the grinding as well. The objective was not to find the grinding conditions which would give the maximum yield of lignin, but rather a practical method to isolate in the neighborhood of 10% of the lignin in the wood.

The wood sawdust was milled without a dispersant and no attempt was made to exclude oxygen. The sawdust was added to the jar already filled about 2/3 full with the steel balls, and the cover was positioned before the jar with the cooling chamber attached was secured in the holder on the vibratory ball mill. The amplitude (i.e., the moment of the eccentric weight) of the vibration was set at a point which seemed to be within the mechanical limitations of the mill. At the completion of grinding, low-boiling petroleum ether was added to the jar to loosen the wood adhering to the jar and the balls. The jar was emptied into a standard sieve fitted with a collecting pan and a tight cover, and the wood powder was removed from the balls with vigorous shaking. Collection of this finely pulverized material could be effected quantitatively by using glass wool to wipe out the jar and the screen. The wood powder and glass wool were centrifuged and allowed to air dry in order to remove the petroleum ether.

The extraction and purification procedure followed the "standard method" of Björkman (34). The finely pulverized wood (and glass wool) was covered with aqueous dioxane (4 ml. water/100 ml. purified dioxane), and the dioxane was renewed occasionally during the extraction period. The combined extract was evaporated to dryness on a rotary vacuum evaporator at 50°C., dissolved in 90% acetic acid, and precipitated into water. The precipitate was centrifuged, and residual water evaporated with a stream of dry air at room temperature. The dry precipitate was dissolved in 1,2-dichloroethane:ethanol (2:1 by volume), centrifuged to remove any undissolved material, and precipitated into anhydrous diethyl ether. The flocculant precipitate was centrifuged and dispersed immediately in fresh ether, being careful to avoid any drying of the precipitate.

This was followed by two more washings with diethyl ether and one with low-boiling petroleum ether, leaving the precipitate stand overnight each time. The precipitate was centrifuged and dried first with a stream of dry air, and then over phosphorous pentoxide and paraffin shavings in a vacuum desiccator. The final product was called milled wood lignin (MWL).

In preliminary attempts to isolate MWL, 6-g. samples (o.d. basis) of the airdry extracted wood containing about 9% moisture were ground 5 hr. in the vibratory ball mill, and various coolants were used to control the grinding temperature. Runs were made using a liquid nitrogen-95% ethanol slurry (-115 to -125°C.) and a 95% ethanol-carbon dioxide slurry (-72°C.) in the cooling chamber, and a third run was made at room temperature without cooling. The three samples of milled wood and a fourth containing unmilled wood were extracted approximately two months with aqueous dioxane, and the MWL was isolated in the usual way. No MWL was obtained from the unground sample, and the yield from each of the other three samples represented only 2% of the Klason lignin.

It was apparent that a 5-hr. grinding period would not release the amount of lignin desired, even at sub-zero temperatures, and the grinding time was increased. The wood was dried in a vacuum desiccator over magnesium perchlorate, and the charge was reduced to 5 g. (o.d. basis) in an attempt to improve the yield. The samples were extracted for three weeks, but most of the lignin was removed during the first few days. Several samples were milled with dry ice (-78°C.) in the cooling chamber, and the yields from 10, 20, and 25-hr. runs were 5, 9, and 13% of the Klason lignin, respectively. The lignin isolated in the

same way from another sample milled at room temperature was designated MWL(RT), and the 11% yield from a 23-hr. run compared quite favorably with the results for the samples processed at -78°C. These preparations were very pale cream-colored powders, but MWL appeared to be a shade lighter than MWL(RT).

The preliminary experimental work suggested that reducing the temperature fails to improve the efficiency of the grinding operation, at least as measured by the accessibility of the lignin to neutral solvents. This might indicate that the solubilization of lignin depends upon rupture of chemical bonds or mechanical depolymerization of the lignin, rather than upon hydrogen bonding or physical phenomena as suggested by Björkman (34).

Large-Scale Isolation of MWL

One hundred and sixty grams of extractive-free aspenwood were processed in the large-scale preparation of MWL using procedures developed in the preliminary experimental work. Each 5-g. sample (o.d. basis) was dried over magnesium perchlorate and milled in a dry state for 25 hr. The temperature was maintained at -78°C. to minimize thermal side effects, and the milled samples were extracted for three weeks with aqueous dioxane. The dioxane extracts were processed according to Björkman's (34) "standard procedure," and the isolated lignin was designated MWL-A. The composite sample was thoroughly mixed with a mortar and pestle, and the combined yield of MWL-A was 3.79 g., or 13.0% of the Klason lignin in the original wood.

Although the "standard" purification procedure is somewhat ambiguous, Björkman (37) noted that a certain amount of lignin was lost when the extracted lignin was dissolved in 90% acetic acid and precipitated into water. The lignin which was solubilized at this point (intermediate lignin) was apparently similar to MWL, but richer in carbohydrate. The isolation and characterization of a portion of this material was undertaken to gain a better understanding of the purification procedure and the relationship of the "intermediate lignin" to MWL-A.

The acetic acid filtrates from 150 g. of milled wood which were formed in the "standard" purification of MWL-A were processed in three equal fractions. Each fraction was evaporated to dryness on a rotary vacuum evaporator at 50°C. The solid residue was extracted with 1,2-dichloroethane:ethanol (2:1) for several days; a large portion of the residue was not dissolved. The extracts were centrifuged, filtered through glass wool, and precipitated into anhydrous diethyl ether. Processing was continued in the usual way, and the isolated lignin was called MWL-B. The three fractions of MWL-B were combined and mixed with a mortar and pestle to obtain a 1.18-g. composite sample which represented 4.3% of the Klason lignin in the original wood.

The isolated lignin preparations were designated "MWL" to indicate that they were extracted originally from milled wood with aqueous dioxane. MWL-A and MWL-B were very light cream-colored powders which represented about 17.3% of the aspenwood Klason lignin. They appeared to be identical when compared visually, and in agreement with Björkman's (34) observation, somewhat lighter than Brauns native lignin (BNL).

CHARACTERIZATION OF LIGNIN PREPARATIONS

The aspen (Populus tremuloides) lignin preparations which were characterized in this investigation are listed in Table II.

TABLE II

ASPEN LIGNIN PREPARATIONS

Sample	Description
MWL(RT)	Lignin preparation extracted with aqueous dioxane from wood milled 23 hr. at room temperature. Purified according to Björkman's (34) "standard" procedure.
MWL-A	Lignin preparation extracted with aqueous dioxane from wood milled 25 hr. at -78°C. Purified according to Björkman's (34) "standard" procedure.
MWL-B	Lignin preparation isolated from the acetic acid filtrate formed during the purification of MWL-A.
BNL	Brauns native lignin from aspenwood. Sample supplied by M. A. Buchanan.

Chemical Analysis of Lignin Preparations

Methods and Results of Analysis

The methoxyl contents of the lignin preparations obtained according to Institute method 18 are shown in Table III.

TABLE III

METHOXYL CONTENT OF LIGNIN PREPARATIONS

Sample	Methoxyl, %
MWL(RT)	20.35
MWL-A	19.30
MWL-B	12.35
BNL	19.29

The carbon-hydrogen and acetyl analyses[1] on MWL-A and MWL-B are shown in Table IV. The transesterification procedure of Whistler and Jeanes (79) was used for the acetyl determination.

TABLE IV

ELEMENTAL ANALYSIS AND ACETYL CONTENT OF LIGNIN PREPARATIONS

Sample	Carbon, %	Hydrogen, %	Ash, %	Acetyl, %
MWL-A	59.60	5.83	None	0.60
MWL-B	52.63	5.83	0.9	1.6

The uronic acid content of the lignin preparations was determined gravimetrically from the carbon dioxide evolution upon refluxing with 12% hydrochloric acid. Details of the procedure can be found in APPENDIX II, and the results are given in Table V.

TABLE V

URONIC ACID DETERMINATION ON LIGNIN PREPARATIONS

Sample	Weight, mg.	Mg. CO_2 Evolved 3 Hr.	Mg. CO_2 Evolved 4.5 Hr.	CO_2, %	Average CO_2, %
Glucurone[a]	4.183	0.974	1.010	23.2	23.2
MWL-A	127.1	1.206	1.263	0.86	0.8
MWL-A[b]	145.1	1.269	1.329	0.79	
MWL-B	102.2	2.114	2.154	1.99	2.0
MWL-B	156.6	3.296	3.364	2.02	
BNL	142.6	0.354	0.450	0.11	0.1

[a] 25% Theoretical CO_2.
[b] Silicone defoamer added.

[1] All carbon-hydrogen and acetyl analyses were run by Huffman Microanalytical Laboratories, P. O. Box 125, Wheatridge, Colorado.

The hydrolysis procedure of Saeman and co-workers (80) was used in the quantitative determination of the polysaccharides associated with the lignin preparations. Approximately 100 mg. of MWL were weighed into a 30-ml. beaker. Three milliliters of cold (10-15°C.) 72% sulfuric acid were slowly mixed with the sample, and the beaker was placed in a 30°C. bath for 1 hr. with occasional stirring. The contents were washed into a 400-ml. beaker with 84 ml. of water and autoclaved for 1 hr. at 15 p.s.i.g. The hydrolyzate was cooled and neutralized by the addition of about 25 g. of freshly-washed Amberlite IR-45 (carbonate form) anion-exchange resin. Enough resin was added to raise the pH to 3.8 in 20-30 minutes. A known amount of ribose was added, and the hydrolyzate was decanted and filtered through glass wool. The filtered hydrolyzate was concentrated on a rotary vacuum evaporator at 45°C. to approximately 5 ml. for chromatographic analysis.

The sugars in the hydrolyzates were separated by paper chromatography. The ethyl acetate:pyridine:water (8:2:1 v./v.) solvent system (81) gave a satisfactory separation for quantitative analysis. In some cases relatively small amounts of sugar were estimated by visual comparison with known quantities of the same sugar after spraying with o-aminobiphenyl phosphate salt in acetic acid (82). This technique was described by Rapson and Morbey (83). Larger amounts of sugar were determined according to the procedure described by Detrick (84). This is a slight modification of the method of Piper and Bernardin (85) in which the sugars are eluted from the chromatogram with an o-aminobiphenyl reagent and determined spectrophotometrically.

The carbohydrate content of the MWL preparations was calculated from the sugar analysis of the acid hydrolyzates. The ratio of individual sugars to ribose was used to establish the amount of each sugar in the total hydrolyzate, and Saeman's (80) correction factors were used to account for the loss of sugars during the hydrolysis. Finally, these values were converted into anhydrosugar units, and the results for MWL-A and MWL-B are shown in Tables VI and VII, respectively.

TABLE VI

CARBOHYDRATE ANALYSIS FOR MWL-A

Anhydrosugar Unit	Mg. in 100 Mg. MWL-A		
	Run 1	Run 2	Average
Galactan[b]	0.16	0.15	0.16
Glucan[b]	0.26	0.28	0.27
Mannan[a]	0.02	0.03	0.03
Araban[b]	0.11	0.09	0.10
Xylan[b]	4.38	4.30	4.34
Rhamnan[a]	0.11	0.13	0.12
		Total	5.02

[a] By visual estimation

[b] By spectrophotometric method

TABLE VII

CARBOHYDRATE ANALYSIS FOR MWL-B

Anhydrosugar Unit	Mg. in 100 Mg. MWL-B
Galactan[a]	0.3
Glucan[a]	0.8
Mannan[a]	0.2
Araban[a]	0.3
Xylan[b]	24.4
Rhamnan[a]	0.2
Total	26.2

[a] By visual estimation

[b] By spectrophotometric method

Discussion of Chemical Analysis

Björkman (34) found that the carbohydrate associated with the MWL preparations appeared to contain hemicellulose sugars, including uronic acid, in about the same proportion as they occur in the wood. A close physical association between lignin and the 5% hemicellulose has been proposed also in the case of aspenwood (86). The aldose fractions of MWL-A and MWL-B are compared with the 5% hemicellulose fraction of aspenwood (86) in Table VIII.

The aldose fractions of MWL-A and MWL-B are predominantly xylan in agreement with the composition of aspenwood hemicellulose, but the glucan, particularly in MWL-A, is higher than expected from the 5%

TABLE VIII

CARBOHYDRATE IN MWL AND 5% HEMICELLULOSE

Anhydrosugar Unit	Composition of Aldose Fraction, %		
	MWL-A	MWL-B	5% Hemicellulose
Galactan	3	1	1
Glucan	5	3	1
Mannan	1	1/2	1
Araban	2	1	2
Xylan	87	94	94
Rhamnan	2	1/2	2

hemicellulose analysis. Timell and associates (87) reported more glucan in certain hardwood species than could be attributed to the cellulose alone, and suggested that this was due to a glucomannan di-heteropolymer. It appears that some of the glucan may be closely associated with the lignin fraction in hardwoods as well.

Recent studies on the polysaccharides of hardwoods (87) indicated that the methyl glucuronoxylans forming the main hemicellulose constituent of all hardwoods contain most of the acetyl groups on the wood. Bouveng, Garegg, and Lindberg (88) isolated a birch xylan with 16.9% acetyl, and these groups appeared to be attached to the xylan residues, mostly in the 3-position, and not to the uronic acid residues. Thus, the predominance of xylan in the carbohydrate fractions of the MWL suggested the possibility of a relatively high acetyl content for these preparations as well. This was borne out by finding that the acetyl

in MWL-A and MWL-B represented about 14 and 7% of the xylan in each preparation, respectively.

The hemicellulose from aspenwood (Populus tremuloides) and other hardwood species contains about 10-11 xylose residues for each uronic acid group (87). Assuming 10 xylose residues per uronic acid, MWL-A and MWL-B should give about 0.15 and 0.82% carbon dioxide, respectively, during the uronic acid determination. In agreement with the infrared analysis (cf. next section), it also indicates that MWL-B should contain 5-6 times as much uronic acid as MWL-A.

Approximately 0.8% carbon dioxide from MWL-A and 2.0% from MWL-B (Table V) were obtained instead. Not only are these values considerably higher than expected, but this also indicates that MWL-B contains only 2.5 (2.0/0.8) times as much uronic acid as MWL-A. The apparently high values may be due in part to oxidation of the carbohydrate as discussed by Björkman (34). The lower ratio (i.e., 2.5 instead of 5-6), however, may indicate carbon dioxide evolution from the lignin[1] itself upon refluxing with 12% hydrochloric acid. Carbon dioxide was liberated from BNL, for example, during the standard uronic acid determination. It appears that this determination may be unsuitable for materials which are predominantly lignin and contain only small amounts of uronic acid. Another experimental approach, possibly quantitative paper chromatography, could prove to be more satisfactory.

The composition of the lignin fraction in MWL-A can be estimated from the elemental analysis given in Table IV, provided a correction

[1] Ultraviolet analysis showed that MWL-A contains about twice as much lignin as MWL-B.

is made for the nonlignin components. This is straightforward for the carbohydrate and acetyl, but somewhat uncertain for the uronic anhydride. The glucuronoxylans are broken down primarily to an aldobiuronic acid composed of methyl glucuronic acid and xylose when hydrolyzed by Saeman's (80) procedure, and part of the xylan escaped detection in this way during the carbohydrate determination (87). On the other hand, the carbon dioxide values may be too high, and a correction based on the aldobiuronic acid would be too large. Because of these uncertainties the carbon dioxide value for MWL-A (Table V) was converted arbitrarily to the equivalent uronic anhydride by multiplying by 4.0, and the corrections for the nonlignin components are summarized in Table IX.

TABLE IX

NONLIGNIN COMPONENTS IN MWL-A

Component	Formula	%
Galactan	$C_6H_{10}O_5$	0.16
Glucan	$C_6H_{10}O_5$	0.27
Mannan	$C_6H_{10}O_5$	0.03
Araban	$C_5H_8O_4$	0.10
Xylan	$C_5H_8O_4$	4.34
Rhamnan	$C_6H_{10}O_4$	0.12
Acetyl	C_2H_3O	0.60
Uronic anhydride	$C_6H_8O_6$	3.2
Total		8.86

From Table IX it was estimated that MWL-A is comprised of approximately 91% lignin and 9% carbohydrate and other material. The calculated

composition of the lignin fraction in MWL-A is compared in Table X with the composition of the lignin reported by Björkman (27) for Populus tremula.

TABLE X

COMPOSITION OF ASPEN LIGNINS

	Composition of the Lignin, %	
	Populus tremuloides	Populus tremula
Carbon	61.15	60.36
Hydrogen	5.87	6.16
Oxygen	32.98[a]	33.00
Methoxyl	21.18	21.44

[a] By difference

The lignin compositions given in Table X were recalculated in order to make a comparison on the basis of phenylpropane building units. This is shown in Table XI.

TABLE XI

COMPOSITION OF ASPEN LIGNINS

Populus tremuloides	Populus tremula
$C_9H_{7.72}O_{2.81}(OCH_3)_{1.39}$	$C_9H_{8.38}O_{2.85}(OCH_3)_{1.43}$

Infrared Absorption Characteristics

The infrared curves for the lignin preparations are presented in Fig. 3, and the strong absorption bands can be correlated with specific functional groups (89). All four samples show a strong hydroxyl group at 3400 cm.$^{-1}$, and the relatively strong bands at 2900 cm.$^{-1}$ can be attributed to aliphatic C—H groups. The absorption band at 1720 cm.$^{-1}$

falls in the normal absorbing region for acid or ester carbonyl, and the band at 1665 cm.$^{-1}$ can be assigned to aldehyde or ketone carbonyl. The two bands about 1600 and 1500 cm.$^{-1}$ in all samples are characteristic of the aromatic nucleus.

Absorbance values for the functional groups were calculated from the absorption spectra in Fig. 3 in the manner described by Kuhn (90). The average transmission values between 1900 and 2200 cm.$^{-1}$, where lignin preparations have no absorption bands, were taken as the intensity of the incident light to correct for scattered radiation. The ratios for the absorbance of each functional group to the absorbance of the aromatic group are shown in Table XII.

TABLE XII

INFRARED FUNCTIONAL GROUP ANALYSIS OF LIGNIN PREPARATIONS

Sample	A_h/A_{ar}	A_{al}/A_{ar}	A_{ec}/A_{ar}	A_{kc}/A_{ar}
MWL-A	0.88	0.48	0.44	0.41
MWL-B	1.65	0.67	1.58	0.53
MWL(RT)	0.76	0.45	0.38	0.40
BNL	0.80	0.30	0.43	0.35

A_h = Absorbance hydroxyl (ca. 3400 cm.$^{-1}$)

A_{al} = Absorbance aliphatic C—H (ca. 2900 cm.$^{-1}$)

A_{ec} = Absorbance ester carbonyl (ca. 1720 cm.$^{-1}$)

A_{kc} = Absorbance ketone carbonyl (ca. 1665 cm.$^{-1}$)

A_{ar} = Absorbance aromatic group (ca. 1600 cm.$^{-1}$)

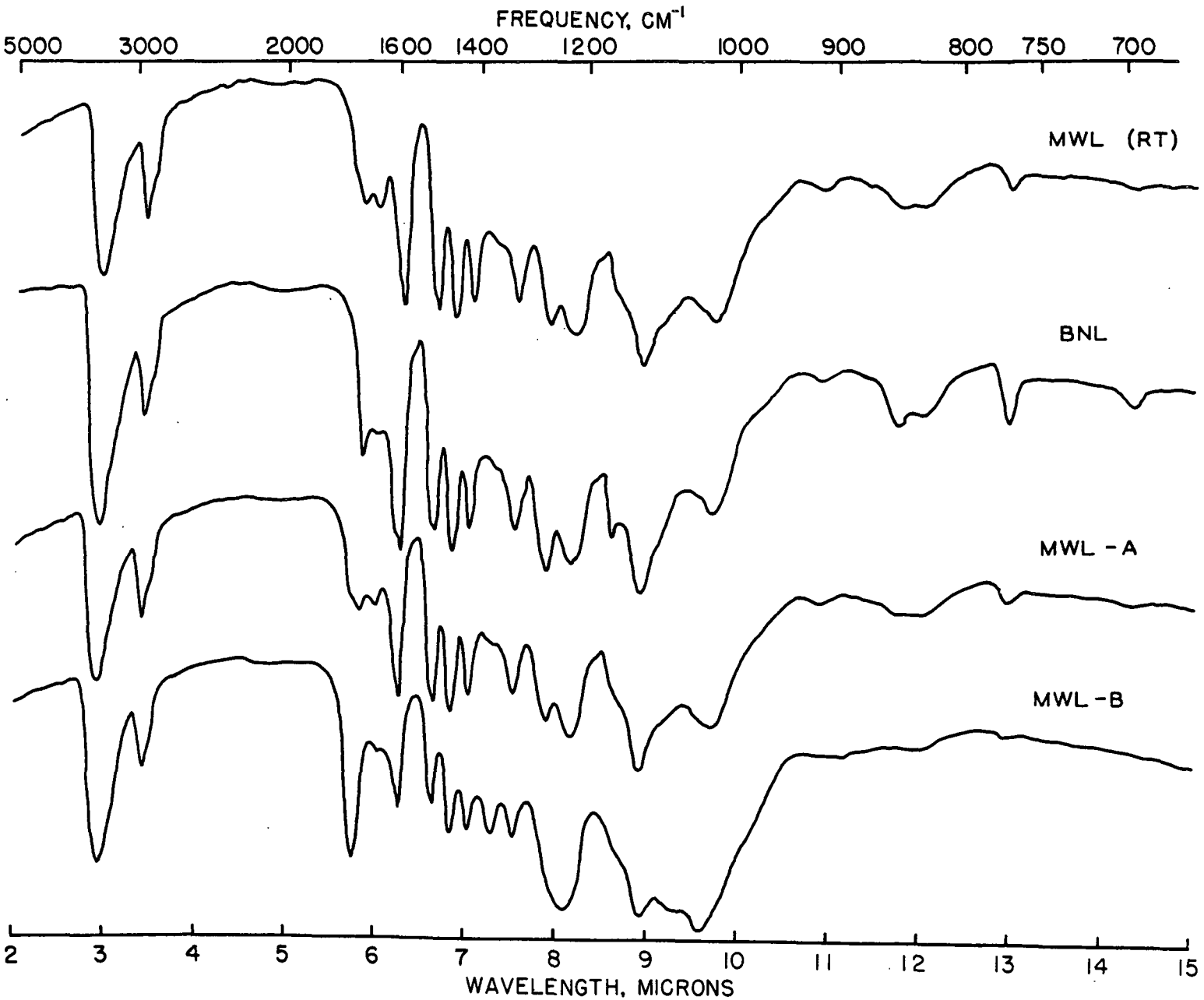

Figure 3. Infrared Absorption Characteristics of Aspenwood Lignin Preparations

Differences among the lignin preparations shown in Table XII can be explained on the basis of their chemical analysis. Some of the aliphatic hydroxyl, aldehyde, or ketone carbonyl, aliphatic C—H, and acid or ester carbonyl groups in the MWL preparations are due to the carbohydrate fraction, whereas the aromatic group is characteristic of the lignin. The ratios shown in Table XII for MWL-B, MWL-A, and MWL(RT) are in accord qualitatively with the estimated amount of carbohydrate in each--45, 10, and 5%, respectively. The lower aldehyde carbonyl and C—H ratios in the case of BNL are consistent with the absence of carbohydrate, but the hydroxyl ratio (0.80) is excessively high. This is probably caused by the presence of p-hydroxybenzoic acid (31) and a higher concentration of benzyl alcohol groups (34) in BNL. p-Hydroxybenzoic acid is apparently responsible for the high ratio (0.43) for the acid or ester carbonyl as well.

An insight into the relative amount of uronic acid in MWL-A and MWL-B can be obtained from Table XII, but it is necessary to estimate the acid or ester carbonyl ratio which is characteristic of the lignin itself. The ratios found for the total acid or ester carbonyl in MWL-A and MWL(RT) were 0.44 and 0.38, respectively. Inasmuch as they contain about 10 and 5% carbohydrate, respectively, it follows that a ratio of 0.32 could be assigned to the lignin. Interestingly enough, this ratio was calculated from the infrared curves reported by Jones (89) and also found to be 0.32 for native spruce lignin.

When the ratios for the total acid or ester carbonyl groups in MWL-A and MWL-B shown in Table XII were reduced by 0.32, the resultant values for the ratios of the carbohydrate acid or ester carbonyl groups in each

were 0.12 and 1.26, respectively. The absorptivity values for the lignin preparations at the far ultraviolet absorption maximum are proportional to the lignin, and consequently to the infrared absorbance values for the aromatic group as well. Absorptivities at the far ultraviolet absorption maximum for MWL-A and MWL-B were 98 and 53, respectively (cf. next section), and the ratio of uronic acid in MWL-B to uronic acid in MWL-A was calculated:

$$(1.26)(53)/(0.12)(98) = 5.7 \qquad (1)$$

Thus, the conclusion was reached from infrared analysis that MWL-B contains 5-6 times as much uronic acid as MWL-A.

Ultraviolet Absorption Characteristics

The ultraviolet absorption characteristics of the lignin preparations were investigated, and freshly distilled methyl cellosolve proved to be a satisfactory solvent for this work. The ultraviolet cut-off with 1-cm. cells and with the maximum sensitivity of the DK-2 recording spectrophotometer was 208 mμ. This permitted a comparison of the various lignins at the point of maximum absorption in the far ultraviolet.

At first an attempt was made to use 10% aqueous methyl cellosolve as a solvent for MWL. The solutions were prepared by dissolving 0.6-0.8 mg. of MWL in 10 ml. of methyl cellosolve and diluting to 100 ml. with distilled water, but the MWL precipitated gradually and almost imperceptibly from solution. Ten per cent methyl cellosolve was a satisfactory solvent for BNL, but even 40% methyl cellosolve was not satisfactory for MWL. This necessitated the change to freshly distilled methyl cellosolve.

The lignin solutions in methyl cellosolve proved to be quite stable--particularly when kept in the dark. One sample showed an absorbance drop from 0.607 to 0.605 (within experimental error) upon standing 24 hr. in the dark, and the absorbance of another sample dropped about 3% after standing one week with exposure to sunlight. However, the samples were usually run within 24 hr. from the time they were prepared.

The absorption maximum of the lignin preparations shifted from 209.5 to 212.5 mμ in going from aqueous (10 and 40%) to nonaqueous methyl cellosolve. The absorptivity of BNL in 10% methyl cellosolve at 209.5 mμ was 106.3 l./g.-cm., whereas that found in freshly distilled methyl cellosolve at 212.5 mμ was 107.1 l./g.-cm. The difference between 106 and 107 was within experimental error, and there did not appear to be any change in the absorptivity accompanying the wavelength shift.

The ultraviolet absorption curves in freshly distilled methyl cellosolve are presented in Fig. 4, and the data can be found in APPENDIX IV. The MWL preparations appear to be almost identical, but BNL lacks a defined absorption maximum near 275 mμ. This was noted before by Pew (59) and attributed to the presence of p-hydroxybenzoic acid.

Table XIII summarizes the absorptivity of the lignin preparations in freshly distilled methyl cellosolve at the 212.5 mμ absorption maximum. The calculated absorptivity for the lignin in MWL-A is slightly higher than the absorptivity of BNL; this might be expected in that any additional amount of p-hydroxybenzoic acid would tend to lower the absorptivity (90a). The absorptivity of MWL-B indicates that it probably contains about 50% lignin.

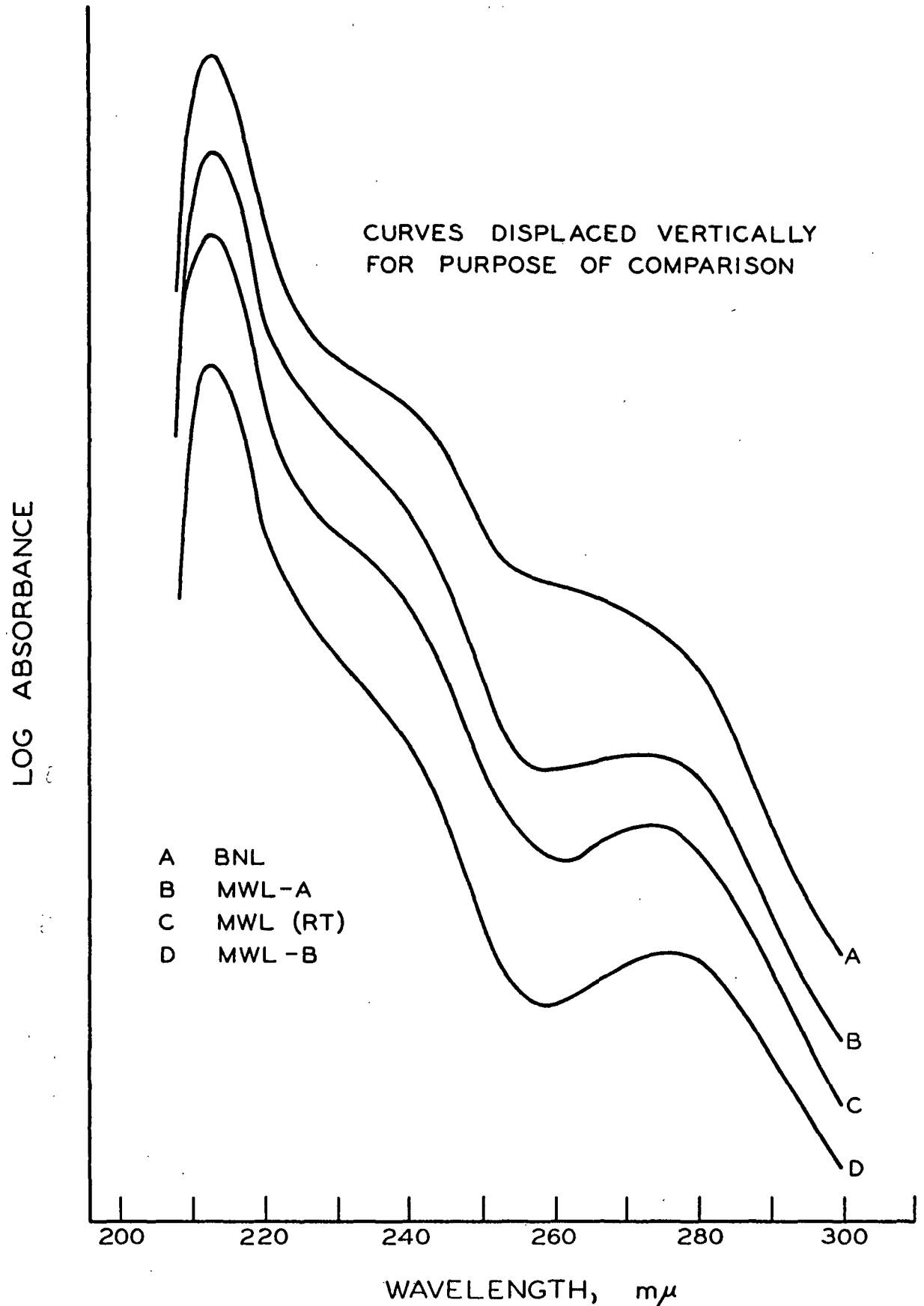

Figure 4. Ultraviolet Absorption Characteristics of Lignin Preparations in Methyl Cellosolve

TABLE XIII

ULTRAVIOLET ABSORPTION CHARACTERISTICS
OF LIGNIN PREPARATIONS

Sample	Absorptivity, l./g.-cm., at 212.5 mμ in Methyl Cellosolve: Original Preparations — Individual Values	Av.	Calculated to 100% Lignin
BNL	106[a], 107	107	107[b]
MWL-A	98, 98, 97	98	108[c]
MWL(RT)	101, 103	102	
MWL-B	53	53	

[a] 10% Methyl cellosolve at 209.5 mμ

[b] Assumes BNL 100% lignin

[c] Calculated on basis MWL-A 91% lignin

METHOXYL BALANCE FOR THE KLASON LIGNIN DETERMINATION

METHOXYL BALANCE ON ASPENWOOD

Pearl and Beyer (5) reported a methoxyl balance on the Klason lignin determination which showed that only 65% of the methoxyl in the wood appeared in the Klason lignin; most of the remaining 35% was in the Klason filtrate. An attempt was made to confirm this observation as indirect evidence for the presence of lignin in the Klason filtrate.

Four 1-g. samples (o.d. basis) of airdry extracted aspenwood were treated individually according to Institute method 13 with 15 ml. of 72% sulfuric acid at 18-22°C. for 3 hr., diluted to 3% acid, and boiled

under reflux for 4 hr. The samples were combined after refluxing and allowed to settle overnight. The Klason lignin was filtered, washed, and dried overnight at 105°C.

The filtrate and washings from the isolation of the Klason lignin were distilled to approximately one-half volume. The distillate was made approximately 2N in sodium hydroxide and redistilled while different fractions were collected until no more methanol came over. The methanol in the distillate was determined quantitatively with chromotropic acid after oxidation to formaldehyde (91).

The partially concentrated Klason filtrate was neutralized by the addition of barium carbonate with stirring until the pH was just above 7.0. The precipitate, barium sulfate with excess barium carbonate, was removed by filtration and washed thoroughly with water. The filtrate and washings were concentrated in a laboratory circulating evaporator to approximately 50 ml. The evaporator was washed with dilute hydrochloric acid to remove the precipitate (mostly barium carbonate) which formed, and the wash water was neutralized to pH 7.0 with sodium hydroxide. It was added to the concentrated filtrate which was taken almost to dryness on a rotary evaporator and dried in an oven at 105°C. The product was a brown sticky material which weighed about 6.4 g. owing to the presence of sodium chloride and other inorganic salts.

The methoxyl content of the Klason lignin and Klason filtrate solids was determined in accordance with Institute method 18. A comparison of the methoxyl balance obtained with that reported by Pearl and Beyer (5) for a different sample of aspenwood is presented in Table XIV. Pearl and

Beyer (5) found that the Klason lignin contained only 65.7% of the methoxyl in the extractive-free wood, whereas the corresponding value was 76.0% in this investigation. This is probably another example of the variability of lignin. Nevertheless, the methoxyl not found in the Klason lignin was still too great to be attributed only to carbohydrate.

TABLE XIV

METHOXYL BALANCE FOR KLASON LIGNIN DETERMINATION ON ASPENWOOD

(Basis: 100 g. ovendry wood meal)

	Weight in Grams	
	Pearl's Data	This Work
Total methoxyl in original wood	5.50	5.41
Extractives	3.2	3.6
Methoxyl in extractives	0.25	0.37
Klason lignin	16.8	17.7
Methoxyl in Klason lignin	3.45	3.83
Methoxyl in Klason lignin filtrate solids	1.37	0.75
Methoxyl in Klason lignin filtrate volatiles	0.41	0.21
Total recovered methoxyl	5.48	5.16

The data in Table XIV were recalculated as shown in Table XV in order to gain a better understanding of the differences between the samples. The methoxyl in the Klason lignin filtrate volatiles was combined with the unrecovered methoxyl to obtain the values shown for the methoxyl split off. It was noted that a methoxyl increase in the Klason lignin was compensated by a decrease in the filtrate, but the methoxyl split off remained about the same.

TABLE XV

METHOXYL BALANCE FOR KLASON LIGNIN DETERMINATION ON ASPENWOOD

(Basis: 100 g. of methoxyl in original wood)

	Weight in Grams	
	Pearl's Data	This Work
Methoxyl in extractives	4.5	6.8
Methoxyl in Klason lignin	62.8	70.8
Methoxyl in Klason lignin filtrate	24.9	13.9
Methoxyl split off	7.8	8.5
	100.0	100.0

Wacek and co-workers (92) showed that only a small amount of methoxyl can be split from lignin during the Klason lignin determination. These investigators (92) also reported that 44% hydrochloric acid at room temperature for 22 hr. caused practically no cleavage of aromatic methyl ethers, but hydrolysis of the methyl ether in 3-methyl glucose was nearly complete. When Bishop (93) hydrolyzed the barium salt of an aldobiuronic acid having a methoxyl/uronic acid molar ratio of 1.1 by heating with 5% sulfuric acid for 12 hr. at 97°C., the aldobiuronic acid isolated from the hydrolyzate was found to have a methoxyl/uronic acid molar ratio of only 0.35. Although the methoxyl in the lignin appeared to be relatively stable, there seemed to be the possibility that methoxyl could be split off almost quantitatively from aspenwood carbohydrate during the Klason lignin determination.

The hypothesized instability of carbohydrate methoxyl during the Klason lignin determination suggested that the 8% methoxyl split off from aspenwood (cf. Table XV) was due chiefly to the carbohydrate. It also suggested that the methoxyl in the Klason filtrate was a measure of the acid-soluble lignin. Therefore, the methoxyl stability of an aldobiuronic acid during the Klason lignin determination was investigated.

METHOXYL STABILITY IN BARIUM ALDOBIURONATE

A crude barium aldobiuronate[1] isolated from extracted E. regnans by dilute acid hydrolysis was used in this investigation. The sample apparently contained a large excess of barium acetate as the chief contaminant. The methoxyl content was 3.58%, and 4.26% carbon dioxide was evolved in the uronic acid determination. This indicated a methoxyl/uronic acid molar ratio of 1.19, but the ultraviolet absorption spectrum and strong Mäule test showed the presence of lignin. A 21% methoxyl content and absorptivity of 106 l./g.-cm. at the 208 mμ absorption maximum were assumed for the lignin, and it was estimated that 30% of the methoxyl was due to the lignin and the other 70% to the uronic acid. This decreased the estimated methoxyl/uronic acid molar ratio for the aldobiuronic acid from 1.19 to 0.83.

Approximately 1.2 g. of the barium aldobiuronate were subjected to the Klason lignin determination in accordance with Institute method 13, and the secondary hydrolysis was carried out under reflux. The sample was diluted with water from 575 ml. (3% sulfuric acid) to 800 ml. and distilled to 400 ml. The distillate was diluted with water to 600 ml.

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[1] Sample supplied by L. E. Wise.

and 50 g. of sodium hydroxide were added before it was redistilled to 200 ml. The methanol in the final distillate was determined with chromotropic acid after oxidation to formaldehyde (91), and the results are presented in Table XVI.

TABLE XVI

METHOXYL STABILITY IN BARIUM ALDOBIURONATE

Basis: 1.2 g. barium aldobiuronate	
Methoxyl in sample, mg.	42.0
Methoxyl from carbohydrate, mg.	29.4
Methoxyl split off, mg.	6.6
Methoxyl loss, % total methoxyl	16
Methoxyl loss, % carbohydrate methoxyl	22

The relatively low value for the methoxyl split off seems to indicate that it may be difficult to infer anything about the methoxyl distribution between carbohydrate and lignin in wood from a methoxyl balance on the Klason lignin determination. However, the aldobiuronic acid used in this investigation was a very crude product, and more work would have to be done before any definite conclusions could be drawn.

INVESTIGATION OF THE ACID-SOLUBLE LIGNIN AND KLASON LIGNIN

THE ACID-SOLUBLE LIGNIN

The low-molecular weight phenols in the aspenwood Klason filtrate were considered part of the acid-soluble lignin, in that they either originated in the lignin, or represented the nonpolysaccharide portion of the extractive-free wood. An investigation of the ether-soluble

fraction of the Klason filtrate was undertaken to study these materials and gain a better understanding of the nature of acid-soluble lignin. Pearl and Beyer (5) reported p-hydroxybenzoic acid in the Klason filtrate from extractive-free aspenwood, and Stanek (32) isolated p-hydroxybenzoic acid and other phenolic materials, which were considered to originate in the lignin, from the water hydrolyzate of extractive-free wood. Therefore, compounds such as vanillic acid, syringic acid, vanillin, and syringaldehyde were expected in addition to p-hydroxybenzoic acid in the Klason filtrate.

Chromatographic Investigation

Eight 1-g. samples (o.d. basis) of airdry extracted aspenwood were subjected to the Klason lignin determination (Institute method 13). The hydrolysis was carried out without reflux, and the isolated Klason lignin was equivalent to 18.17% of the ovendry wood. The Klason filtrates were combined and shaken three times with equal volumes of diethyl ether. The extract was dried over anhydrous sodium sulfate and concentrated to approximately 50 ml. for chromatographic analysis.

The procedures described by Stanek (32) were used in the chromatographic investigation of the ether extract. The extract was spotted along with authentic samples on Whatman No. 1 filter paper and developed by the descending method. The extracts were examined for aldehydes using butanol saturated with 2% aqueous ammonia and n-butyl ether saturated with water as developers. The ethyl acetate-pyridine-water (8:2:1) solvent system (81) was employed specifically for the separation of furfural and hydroxymethylfurfural. The developers used in the identification of

acids were benzene saturated with formic acid and butanol-pyridine-water (10:3:3). The chromatograms were examined under ultraviolet light, and sprayed with 2,4-dinitrophenylhydrazine, p-anisidine hydrochloride (93), phloroglucinol, diazotized p-nitroaniline, bis-diazotized benzidine, and Mäule test reagents.

The chromatographic investigation demonstrated the presence of vanillic acid, syringic acid, and p-hydroxybenzoic acid in the ether extract. The amount of p-hydroxybenzoic acid appeared to be much larger than that of the other two acids. The only aldehyde found in the extract was identified as hydroxymethylfurfural. If vanillin and syringaldehyde were formed, they may have condensed with the lignin under the conditions of the Klason lignin determination (51). Furfural produced by the action of sulfuric acid on the xylans may have condensed with the lignin, or it may have steam distilled as suggested by Marth (47).

Quantitative Determination of Phenolic Acids

The aspenwood Klason filtrate was examined quantitatively for p-hydroxybenzoic acid, vanillic acid, and syringic acid. The filtrates from five separate determinations (Institute method 13) on a total of 5.08 g. (o.d. basis) of extracted aspenwood were combined and extracted with ether for about 40 hours in an air-agitated continuous extractor. The ether extract was dried over anhydrous sodium sulfate and concentrated to 100 ml.

The procedure described by Pearl, et al. (95) was used for the quantitative determination of the acids present in the Klason filtrate.

A known amount of the ether extract was streaked on the top of a sheet of Whatman No. 1 filter paper and developed with benzene saturated with formic acid. A guide strip was cut from the developed chromatogram and sprayed with diazotized p-nitroaniline to locate the bands containing the acids. Each band was cut from the chromatogram and eluted with 95% ethanol, and the acids in the eluates determined spectrophotometrically. The results presented in Table XVII show the predominance of p-hydroxybenzoic acid as suggested by the qualitative chromatograms.

TABLE XVII

PHENOLIC ACIDS IN THE ASPENWOOD KLASON FILTRATE

(Basis: 1 gram ovendry wood meal)

Acid	Weight, mg.
Vanillic	0.2
Syringic	0.4
p-Hydroxybenzoic	2.0

THE KLASON LIGNIN

A 0.2500-g. portion of a Klason lignin[1] was subjected to the Klason lignin determination (Institute method 13), and the weight of the secondary Klason lignin was 0.2363 g. The resultant Klason filtrate (the secondary filtrate) was diluted with water to 1000 ml. A 100-ml. aliquot was removed for spectroscopic analysis and the rest of the secondary filtrate was extracted with diethyl ether. The ether extract was dried over anhydrous sodium sulfate and concentrated to approximately 10 ml. on

[1] Klason lignin isolated during chromatographic investigation of acid-soluble lignin.

a rotary evaporator. Another 0.5-g. portion of a Klason lignin[1] was extracted in a beaker with 25 ml. of 95% ethanol for several hours at room temperature, and the extracts were compared by means of paper chromatography in the manner previously described. The alcohol extract of the Klason lignin contained no detectable phenolic acids, whereas the ether extract of the secondary filtrate contained vanillic acid, syringic acid, and p-hydroxybenzoic acid. Again there was considerably more p-hydroxybenzoic acid than either vanillic acid or syringic acid.

The finding of vanillic, syringic, and p-hydroxybenzoic acids in the ether extract of the secondary filtrate, but not in the alcohol extract of the Klason lignin, supported Stanek's (32) hypothesis that these acids can be obtained through hydrolysis of the lignin. The predominance of p-hydroxybenzoic acid also suggested that a large portion of the p-hydroxybenzoic acid in the extractive-free wood may be retained in the Klason lignin.

Presumably, most of the p-hydroxybenzoic acid in the extractive-free wood is esterified with the lignin (31), and can be removed by alkaline hydrolysis. However, it is possible that some of the p-hydroxybenzoic acid cannot be removed completely by acid hydrolysis during the Klason lignin determination due to equilibrium and rate considerations. Another portion might be liberated during the Klason lignin determination and condensed with the lignin (96) in such a way that it could no longer be liberated by alkaline hydrolysis. Therefore, p-hydroxybenzoic acid in the Klason lignin was estimated from the

[1] Klason lignin isolated during methoxyl balance for Klason lignin determination.

difference between the amount in the Klason filtrate and the amount in the alkaline hydrolyzate from the wood itself.

The ether-extracted Klason filtrate from the quantitative determination of phenolic acids was neutralized with sodium hydroxide and concentrated to 1500 ml. in a circulating evaporator. The concentrated filtrate was made 1N in sodium hydroxide and refluxed for 8 hr. with vigorous stirring. After acidification with sulfuric acid, the filtrate was extracted with diethyl ether for 40 hr. in a continuous extractor, and the extract was dried over anhydrous sodium sulfate and concentrated to 100 ml. The extract was examined chromatographically in the usual way and found to contain only a trace of p-hydroxybenzoic acid.

A 50-g. sample (o.d. basis) of extractive-free aspenwood was hydrolyzed with 1500 ml. of boiling 1N sodium hydroxide for 8 hr. with vigorous stirring. The alkaline hydrolyzate was acidified and extracted with ether for 40 hr. in an air-agitated continuous extractor, and the ether extract was dried over anhydrous sodium sulfate and concentrated to 100 ml. The p-hydroxybenzoic acid in the ether extract was determined quantitatively as previously described. The 280 mg. of p-hydroxybenzoic acid found in the total extract corresponded to 5.6 mg. from each gram of wood.

Only 2.0 mg. of the estimated 5.6 mg. of p-hydroxybenzoic acid per gram of extractive-free wood were found free in the Klason filtrate (Table XVII). Inasmuch as only a trace was freed by alkaline hydrolysis of the Klason filtrate, most of the remainder was presumed to be condensed or esterified with the Klason lignin. This indicated that approximately

64% of the p-hydroxybenzoic acid was left with the Klason lignin and suggests, therefore, that the Klason lignin from extractive-free aspenwood contained about 2% p-hydroxybenzoic acid.

This was confirmed later by Pearl and Beyer (97). When an isolated Klason lignin from aspenwood was fused with potassium hydroxide for five minutes at 175°C., approximately 2% of the lignin was isolated as p-hydroxybenzoic acid. This also indicated that the p-hydroxybenzoic acid was not condensed with the lignin during the Klason lignin determination. Apparently, the p-hydroxybenzoic acid moiety is a significant part of both the acid-soluble lignin and the Klason lignin of aspen.

THE FAR ULTRAVIOLET FOR THE MEASUREMENT OF ACID-SOLUBLE LIGNIN

INTRODUCTION

The determination of acid-soluble lignin in the Klason filtrate from ultraviolet absorption measurements depends upon the characteristic absorption of the substituted benzene nucleus in lignin. The furan-type carbohydrate hydrolysis products interfere with measurements in the near ultraviolet, but recent work (73) showed that the 205 mμ absorption maximum for lignin could be used as an empirical tool for estimating lignin in the acid hydrolyzates of unbleached pulps. The work presented here was undertaken to establish the feasibility of using the far ultraviolet to obtain absorption measurements characteristic of the lignin in the Klason filtrate without the customary interference from carbohydrate degradation products.

ABSORPTION CHARACTERISTICS OF THE KLASON FILTRATE

The Effect of Sulfuric Acid

The absorption measurements on the Klason filtrates were made against dilute sulfuric acid solutions of approximately the same acid concentration. Any difference between the acid concentrations of the blank and the sample causes an error in the measured absorbance values. In order to estimate the magnitude of this error, a 3% sulfuric acid solution was run against water on the DK-2 spectrophotometer, and the calculated absorptivity curve for the sulfuric acid is presented in Fig. 5.

In the procedure for the Klason lignin determination, 72% sulfuric acid was added dropwise to the wood from a buret (stopcock lubricated only with acid), and the volume added was 15.0 $\pm$ 0.1 ml. The temperature of the acid was 15 $\pm$ 5°C. The blank was prepared in the same way, and for the maximum temperature and volume differences expected between the sample and the blank, the error calculated from the absorptivity in Fig. 5 was less than 0.003 absorbance units at 200 mμ. This indicates that errors due to variations in the concentration of sulfuric acid were insignificant, and supports Marraccini and Kleinert's (73) observation that there is no appreciable absorption by sulfuric acid between 204 and 220 mμ.

The Effect of Ether Extraction

A 1-g. sample of airdry aspenwood was treated with 15 ml. of 72% sulfuric acid for 3 hr. at 20°C. The sample was diluted with water to

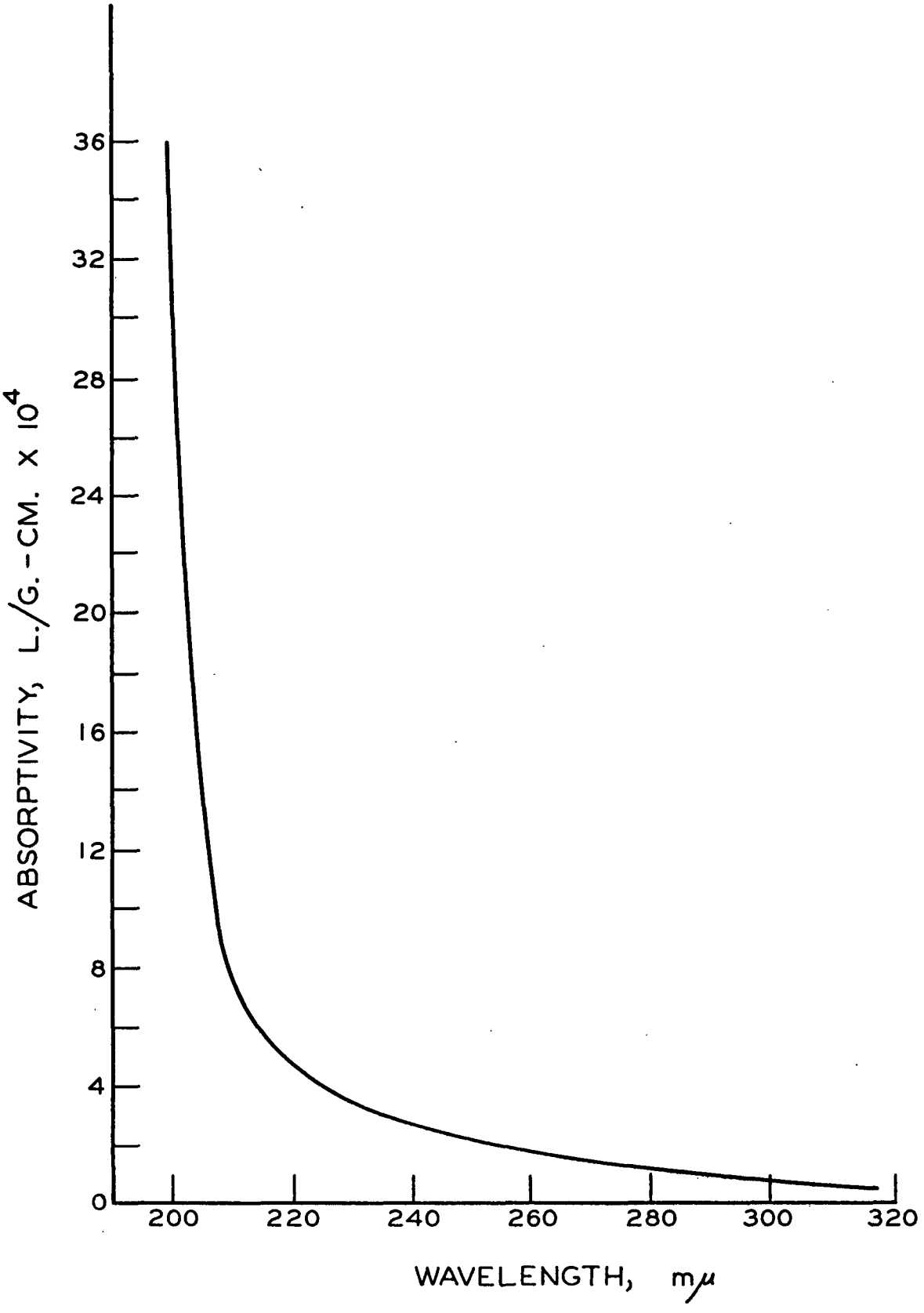

Figure 5. Absorptivity of Sulfuric Acid

575 ml. (3% sulfuric acid) and boiled 4 hr. under reflux. The hydrolyzed sample was cooled and filtered (without further dilution) through a Selas crucible to remove the Klason lignin. A portion of the resultant Klason filtrate was extracted three times with equal volumes of diethyl ether and heated at 35°C. for ten minutes on a rotary evaporator to remove the residual ether. The ultraviolet absorption curves of the Klason filtrate, before and after ether extraction, were determined on a DK-2 spectrophotometer using a 3% sulfuric acid solution as blank. The absorption curves presented in Fig. 6 have been adjusted to correspond to 1.000 g. (o.d. basis) of the original wood.

If the light absorption at the far ultraviolet absorption maximum is attributed only to acid-soluble lignin, approximately 20% of the total lignin in the Klason filtrate can be extracted with ether. The large absorbance drop at the 280 mμ absorption maximum, however, indicates the removal of the carbohydrate hydrolysis products as well.

The Effect of Secondary Hydrolysis

In order to study the effect of secondary hydrolysis on the formation of acid-soluble lignin and the ultraviolet absorption characteristics of the Klason filtrate, the absorption spectrum of the Klason filtrate was determined after refluxing for different periods in the absence of the Klason lignin. A 1-g. sample of the airdry aspenwood was treated with 15 ml. of 72% sulfuric acid for 3 hr. at 20°C. The sample was diluted with water to 575 ml. and filtered immediately through a Selas crucible (no. 4010--maximum pore size $\cong$ 10 μ). Two separate portions of the filtrate

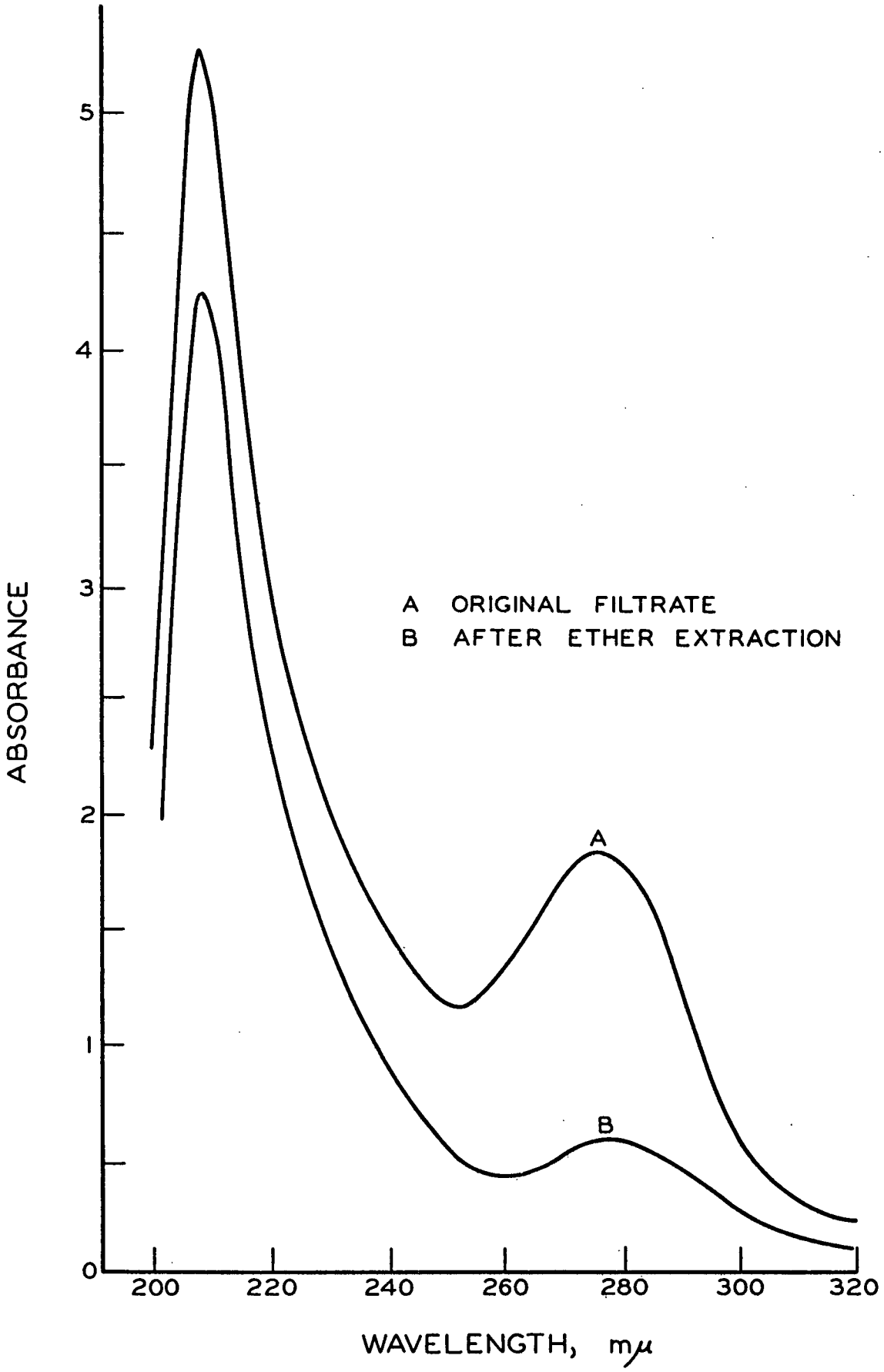

Figure 6. Ultraviolet Absorption Characteristics of Aspenwood Klason Filtrates

were refluxed for 2 and 4 hr., respectively. After standing overnight, all three samples were refiltered. The absorption curves presented in Fig. 7 were determined against 3% sulfuric acid on a DK-2 spectrophotometer, and adjusted to correspond to 1.000 g. (o.d. basis) of the original wood.

The filtrate which was not refluxed had the highest absorbance at 208 mμ; some colloidal lignin could not be removed until the filtrate was boiled. There was little difference between the absorbance values for the 2 and 4-hr. samples at 208 mμ, which suggested that carbohydrate degradation products have only a small effect upon light absorption in the far ultraviolet.

A comparison of Fig. 6 and 7 shows that the absorbance values at 208 mμ are about the same whether or not the filtrate is refluxed with Klason lignin. As a matter of fact, the absorbance appears to be as high at 208 mμ if the filtrate is not refluxed. This indicates that the acid-soluble lignin is probably formed during the initial treatment with 72% sulfuric acid, and not during the secondary hydrolysis with dilute acid.

The Beer's Law Relationship

A 1-g. sample of airdry extractive-free aspenwood was subjected to the Klason lignin determination (Institute method 13); the secondary hydrolysis was carried out in a beaker covered with a watch glass. The Klason filtrate was diluted to 1000 ml., and the Klason lignin corresponded to 18.13% of the ovendry wood.

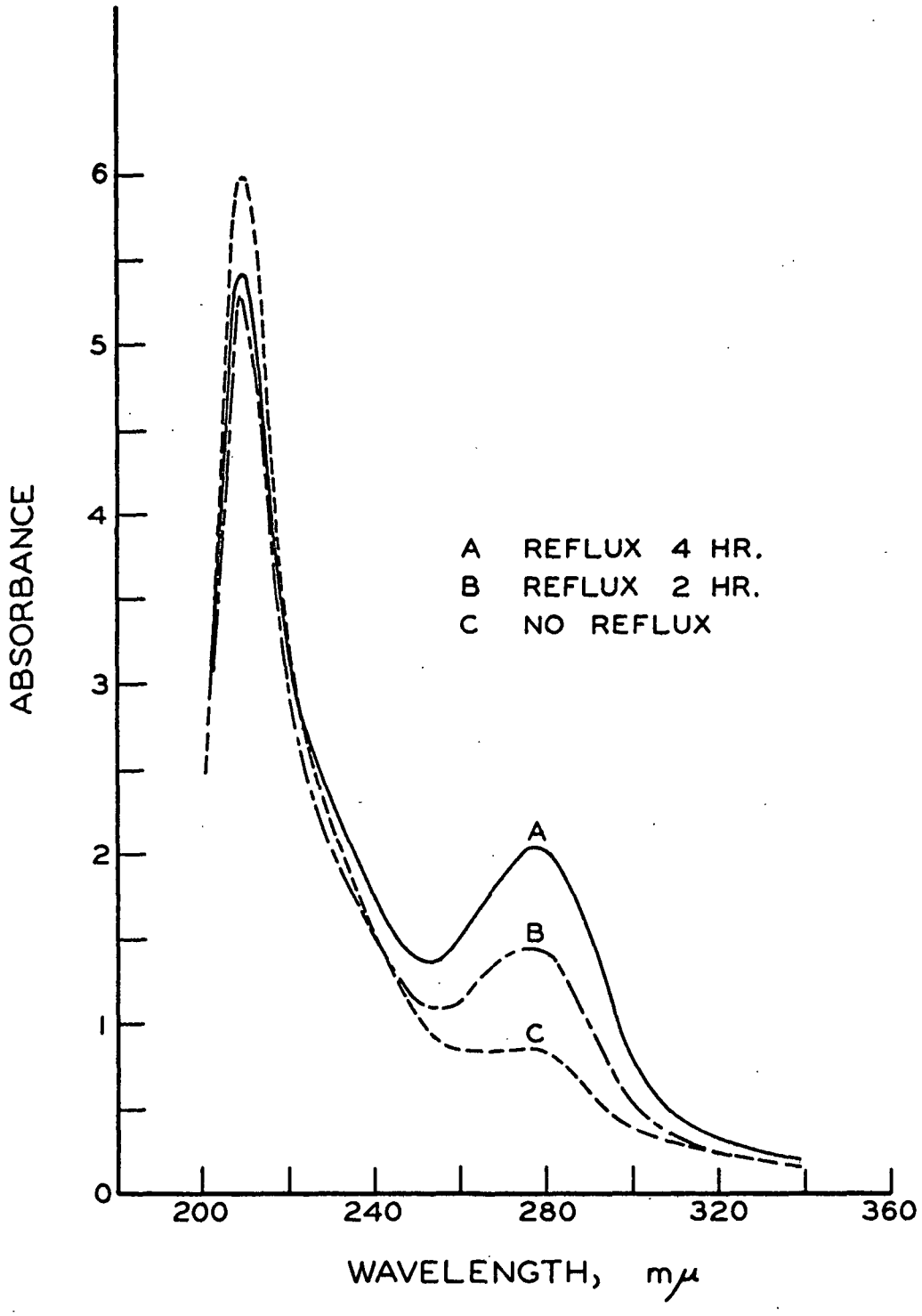

Figure 7. Ultraviolet Absorption Characteristics of Aspenwood Klason Lignin

Samples for ultraviolet absorption measurements were prepared by dilution of the Klason filtrate with 1 1/2% sulfuric acid (15 ml. of 72% sulfuric acid diluted to 1000 ml.), and run against 1 1/2% sulfuric acid on the DK-2 spectrophotometer. The results presented in Table XVIII correspond to 1.000 g. of ovendry wood, and the concentration values shown denote the milliliters of the original Klason filtrate (1000 ml.) which were diluted to 100 ml.

TABLE XVIII

ABSORBANCE VALUES FOR THE KLASON FILTRATE

Concentration, %	$\lambda_{max.}$	$\underline{A}$ at $\lambda_{max.}$	$\underline{A}$ at 208 mμ
18	208.0	0.557	0.557
30	208.4	0.900	0.897
42	209.1	1.271	1.253

The position of the absorption maximum depended to a certain extent upon the concentration of acid-soluble lignin. The wavelength appeared to approach 207.5 mμ as a limiting value in dilute solutions and increased beyond 209 mμ in more concentrated solutions. This indicated some interaction of the lignin in solution, and in this connection it should be noted that Kleinert and Joyce (69) suggested that the dissolution state may have some effect upon the absorption characteristics of lignin preparations.

The absorbance values at 208 mμ (Table XVIII) which are plotted in Fig. 8 show a linear relationship between absorbance and concentration in accordance with Beer's law. Thus, the Beer's law relationship may be

used to calculate the concentration of acid-soluble lignin in the Klason filtrate from absorbance measurements at 208 mμ. If the concentration is adjusted to keep the absorbance values below 1.0, the errors produced by a shift in the absorption maximum can be kept less than the actual experimental errors.

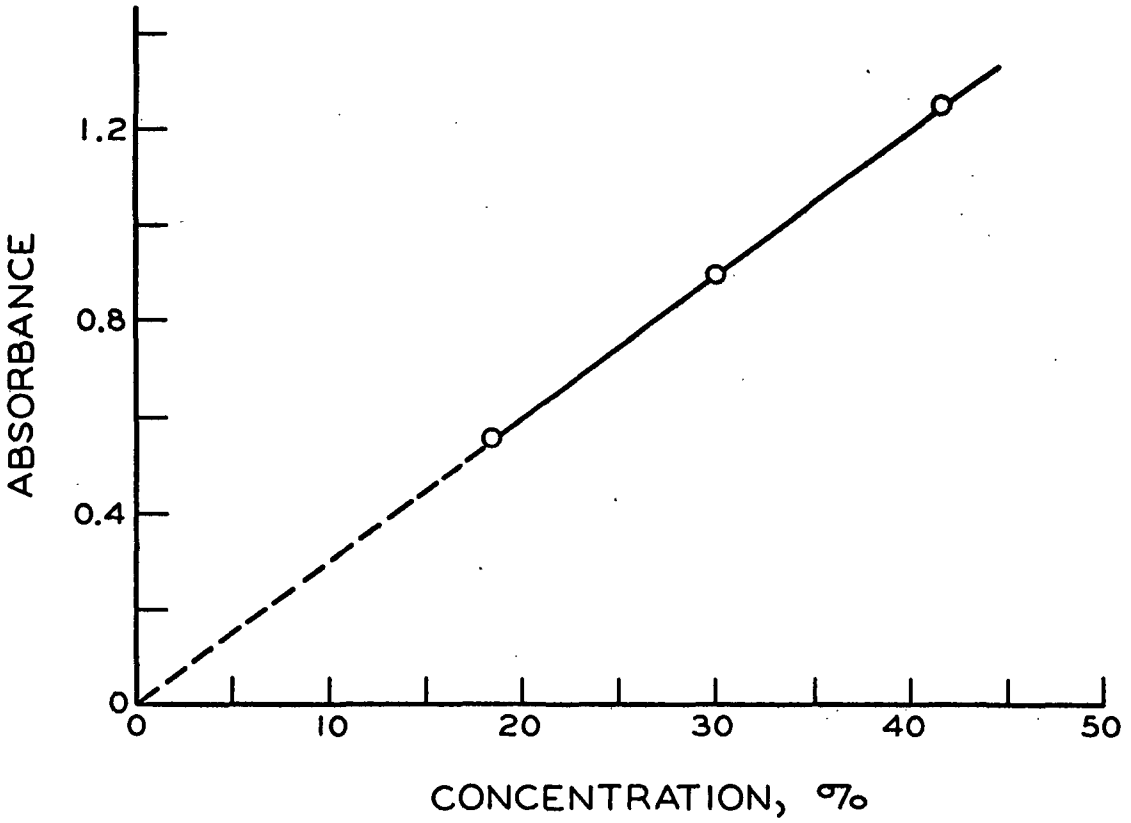

Figure 8. Beer's Law Plot for Klason Filtrate

Stability of the Ultraviolet Absorption

The Klason filtrate absorbance at 208 mμ decreased about 1-2% in the first three days, and about 9% after standing three months. This can probably be explained by a tendency for the acid-soluble lignin to flocculate and precipitate from solution. Although the filtrates are relatively stable, measurements were usually made as soon as possible.

ABSORPTION CHARACTERISTICS OF GLUCOSE AND XYLOSE HYDROLYSIS PRODUCTS

A 1.000-g. sample of glucose and a 0.1000-g. sample of xylose were treated with 15 ml. of 72% sulfuric acid for 3 hr. at 20°C. The samples were diluted with water to 575 ml. and refluxed for 6 hr. Portions of each hydrolyzate were removed periodically, and the ultraviolet absorption curves were run against 3% sulfuric acid on the DK-2 spectrophotometer. The curves for glucose and xylose are presented in Fig. 9 and 10, respectively.

EFFECT OF CARBOHYDRATE ON THE ULTRAVIOLET ABSORPTION OF THE KLASON FILTRATE

The contribution of carbohydrate to the ultraviolet absorption of the Klason filtrate was estimated from the hydrolysis curves for glucose and xylose (Fig. 9 and 10). The carbohydrate fraction in 1 g. of extractive-free aspenwood was estimated to be equivalent (cf. APPENDIX III) to the combined effect of 0.524 g. of glucose and 0.267 g. of xylose. The predicted absorbance for the carbohydrate was obtained, therefore, by adding the product of 0.524 and the absorbance in Fig. 9 to the product of 0.267 and 10 times the absorbance in Fig. 10. This was done throughout the

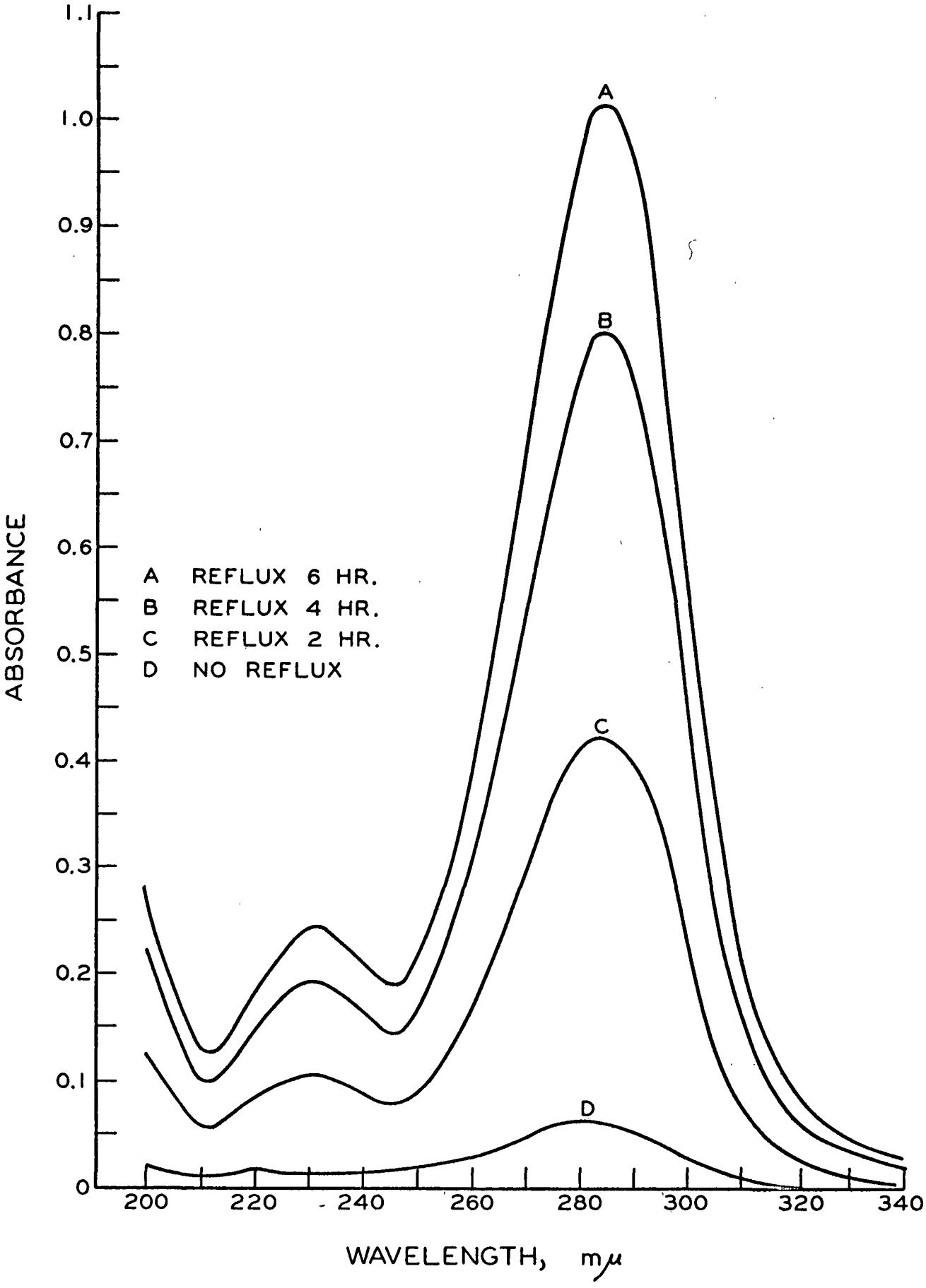

Figure 9. Ultraviolet Absorption Characteristics of Glucose Hydrolyzate

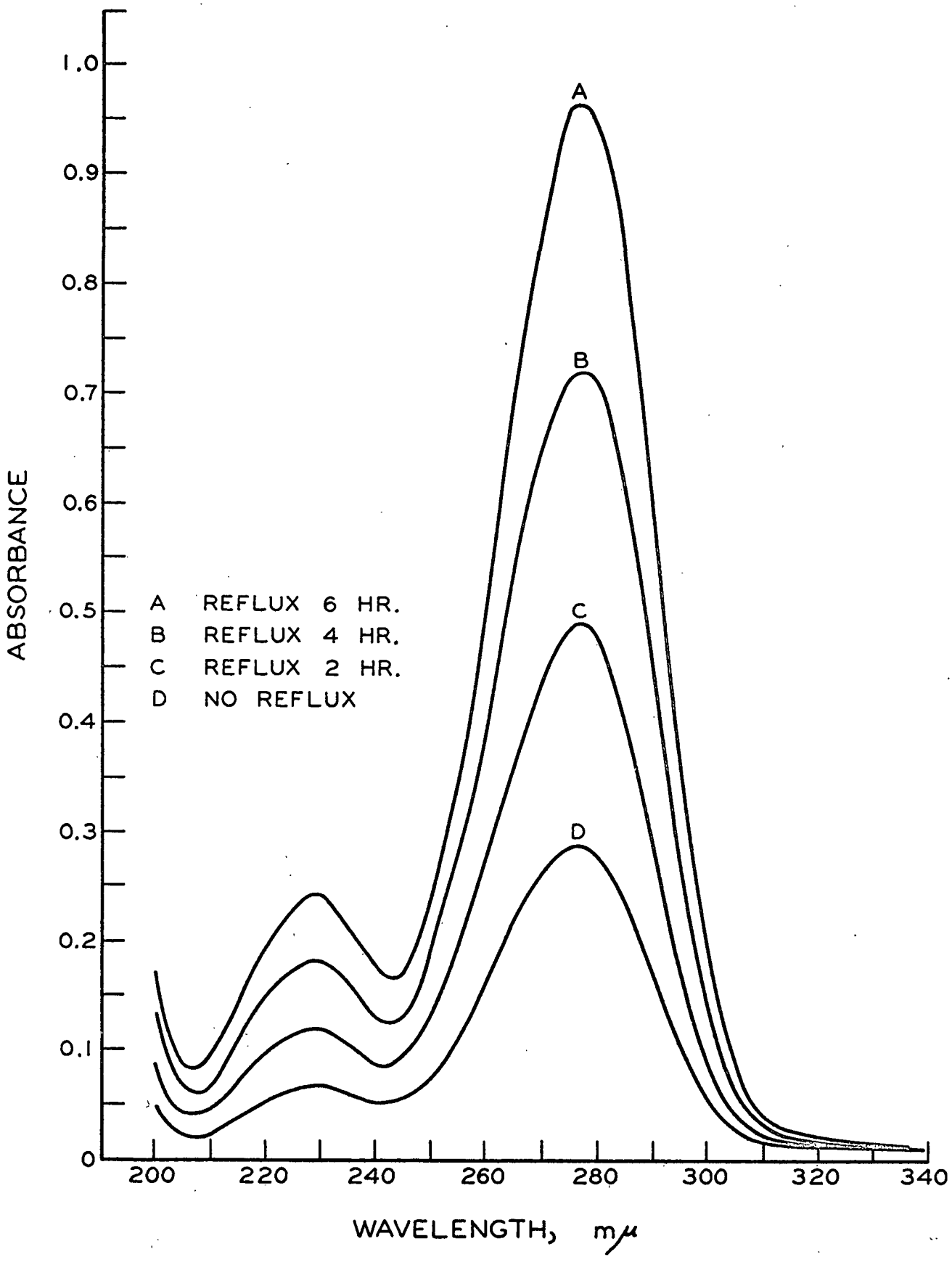

Figure 10. Ultraviolet Absorption Characteristics of Xylose Hydrolyzate

ultraviolet spectrum, and the resulting curve is compared in Fig. 11 with the previous curve (Fig. 6-A) for the Klason filtrate.

The absorbance predicted for the carbohydrate at 280 mμ was higher than the absorbance actually found for the Klason filtrate. The same phenomenon was observed by Browning and Bublitz (57) and attributed to absorption of furan-type compounds by the Klason lignin. A condensation of carbohydrate degradation products with the lignin appears to be the only logical explanation in this case as well.

The most significant feature of Fig. 11 is the difference at 208 mμ between the absorbance of the Klason filtrate and the absorbance predicted for the carbohydrate. Not only does the lignin exhibit its maximum absorption at this point, but the absorption of carbohydrate hydrolysis products is also at a minimum. Undoubtedly, carbohydrate interference in the determination of acid-soluble lignin can be minimized at 208 mμ.

DISCUSSION

This investigation indicated the feasibility and desirability of determining acid-soluble lignin by means of absorption measurements in the far ultraviolet. In addition, it demonstrated the existence of acid-soluble lignin in the aspenwood Klason filtrate.

ULTRAVIOLET STANDARD FOR ACID-SOLUBLE LIGNIN

INTRODUCTION

The selection of an ultraviolet standard for acid-soluble lignin stands out as the second major problem, and perhaps the most difficult,

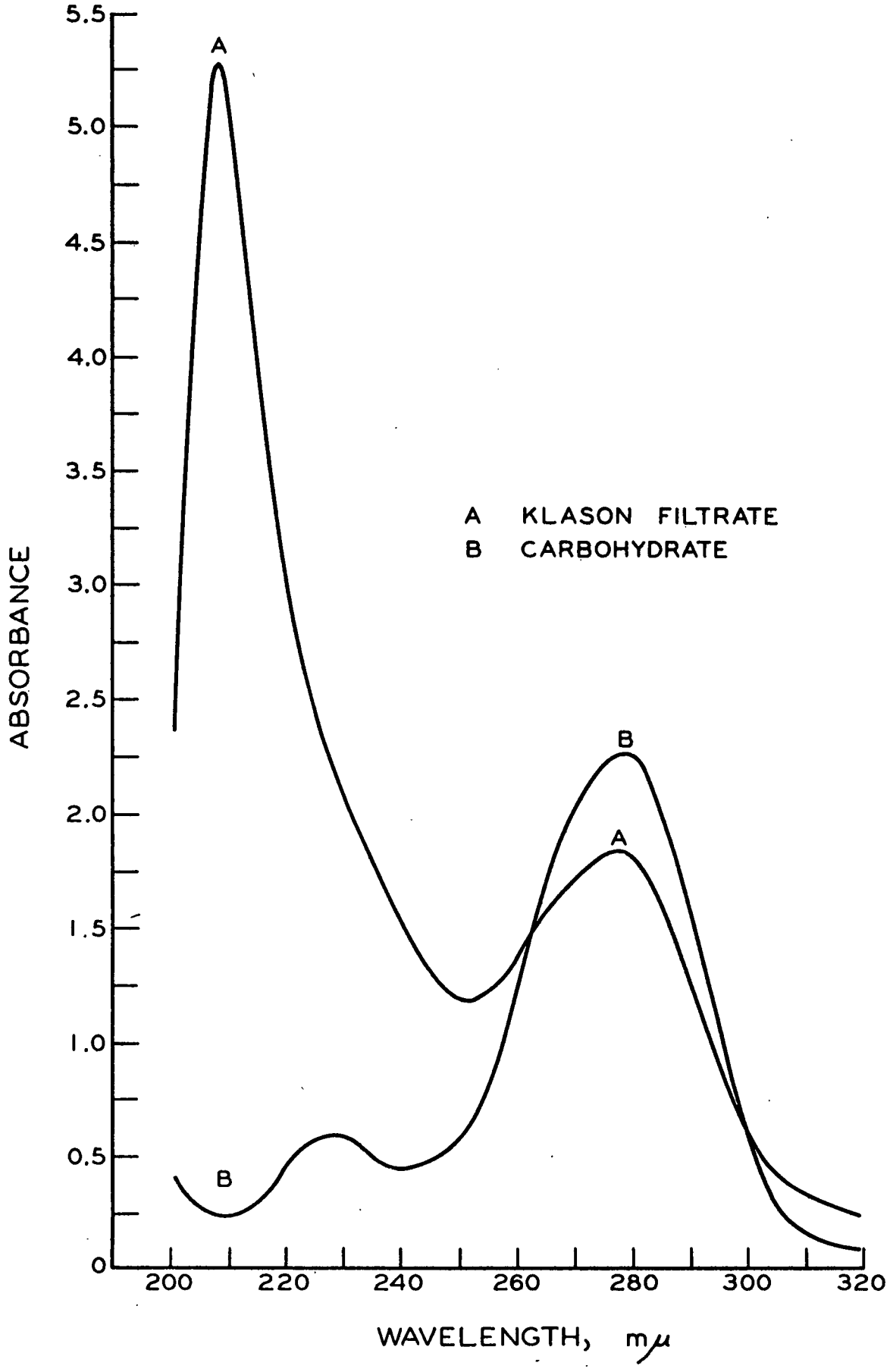

Figure 11. Comparison of the Ultraviolet Absorption Characteristics of the Klason Filtrate and Carbohydrate Hydrolysis Products

in the spectrophotometric determination of lignin in the Klason filtrate. The hypothesized similarity between MWL-A and protolignin suggested that MWL-A might be a suitable standard for acid-soluble lignin, provided there are no drastic changes in the absorption characteristics of the protolignin upon transformation to acid-soluble lignin. It also suggested that subjecting MWL-A to the Klason lignin determination might result in a better understanding of the absorption characteristics and formation of acid-soluble lignin from the wood itself.

MWL AS A STANDARD FOR ACID-SOLUBLE LIGNIN

The ultraviolet absorption characteristics of acid-soluble lignin in the Klason filtrate cannot be compared directly with other lignin preparations owing to the presence of carbohydrate degradation products. However, only 20% of the acid-soluble lignin was removed on ether extraction of the Klason filtrate. Inasmuch as there was concomitant removal of most of the carbohydrate degradation products, the absorption curve of the extracted filtrate was characteristic of approximately 80% of the acid-soluble lignin. It is compared in Fig. 12 with the absorption curves of MWL-A and an aspen lignosulfonate[1] isolated from NSSC spent liquor.

In discussing the ultraviolet spectroscopy of lignin, Aulin-Erdtman (26) concluded that sulfonation of lignin probably takes place in the non-aromatic parts of the molecule and that lignosulfonates should have curves similar to lignin as it exists in the wood:

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[1] NSSC lignosulfonate supplied by M. A. Buchanan.

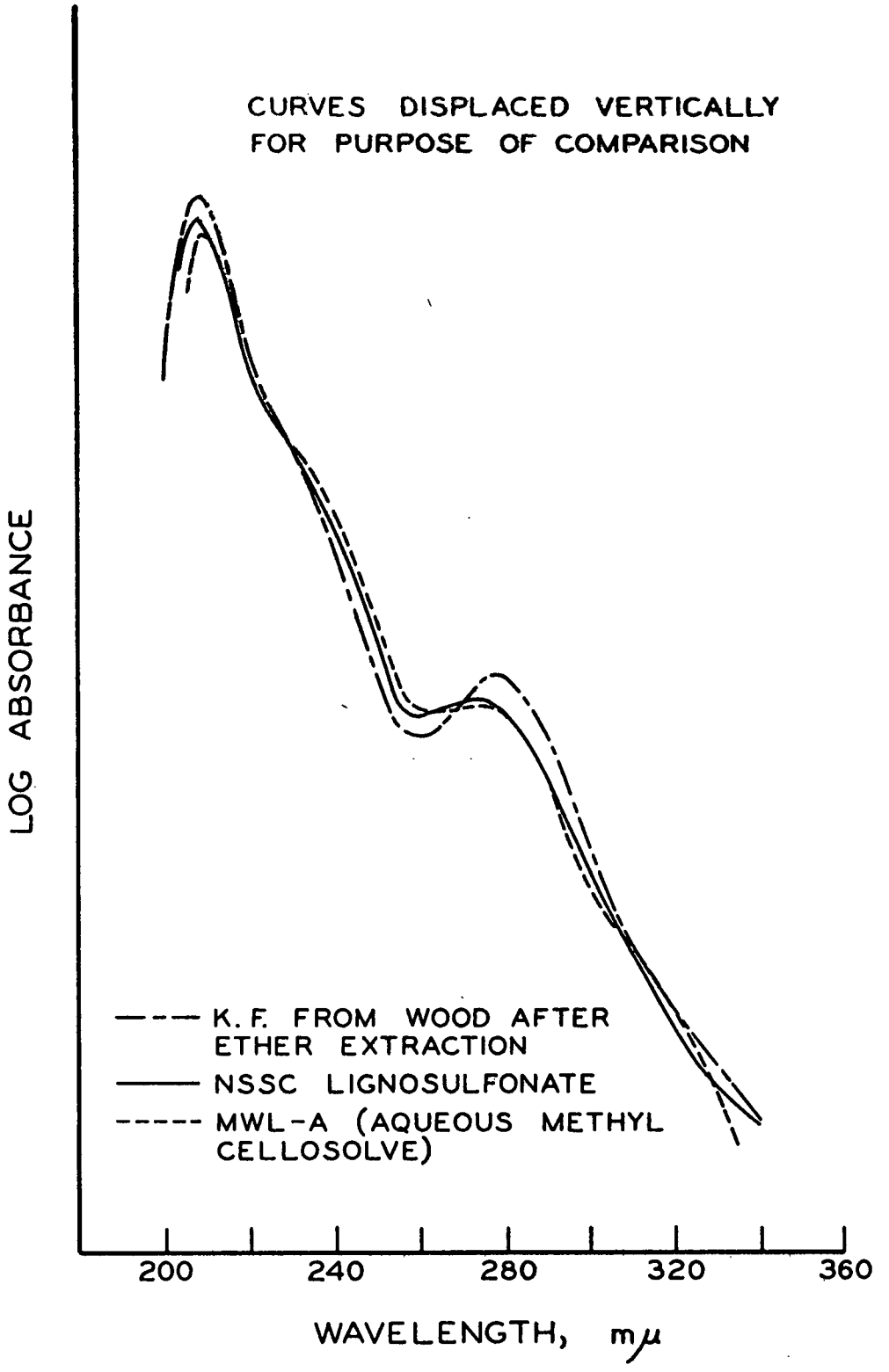

Figure 12. Ultraviolet Absorption Characteristics of Aspen Lignin Preparations

> "Now the question is how to choose in order to find the preparations (lignin) with the fewest and least severe alterations from the original structure. Some guidance is given by the color of the preparations. Since the lignin in the wood is very light or colorless, dark preparations are bound to have undergone changes, these being more severe the darker the color of the material. The ultraviolet absorption curves of the dark lignin preparations are always flat, whereas the light ones show steep curves with sharper maxima and minima, their curves being more similar to those of pure aromatic substances. In this respect lignosulphonic acids are outstanding among the lignin preparations."

The absorption curves of MWL-A and the NSSC lignosulfonate (Fig. 12) are almost identical in agreement with Aulin-Erdtman's (26) hypothesis about the spectra of lignosulfonates. It is surprising, however, that the acid-soluble lignin in the Klason filtrate from wood (or at least 80% of it) has an absorption curve which is steep, sharp, and distinct, and remarkably similar to the lignosulfonate and MWL-A. This seems to indicate that the acid-soluble lignin was not severely altered during the Klason lignin determination. Apparently, MWL-A(or a lignosulfonate) can be considered a satisfactory ultraviolet standard for most of the acid-soluble lignin in the aspenwood Klason filtrate.

KLASON LIGNIN DETERMINATION ON ISOLATED LIGNIN PREPARATIONS

Samples of MWL-A and BNL were subjected to the Klason lignin determination (Institute method 13). The secondary hydrolysis was carried out without reflux, and a sample size was employed which gave about the same amount of Klason lignin as a 1-g. sample of wood. The Klason filtrates were diluted to 1000 ml., and ultraviolet absorption measurements were made against 1 1/2% sulfuric acid on the DK-2 spectrophotometer. A

parallel experiment was run previously (cf. p. 57) on an isolated Klason lignin, and the data are summarized in Table XIX.

TABLE XIX

KLASON LIGNIN DETERMINATION ON LIGNIN PREPARATIONS

Sample	Weight, mg.	Klason Lignin, mg.	Methoxyl in K.L., %	Filtrate[a] Absorbance
MWL-A	229.3	182.9	21.3	1.290
MWL-A	207.6	166.0	21.3	1.146
BNL	206.4	183.3	20.3	0.962
K.L.[b]	250.0	236.3	--	0.588

[a] Absorbance at 208 mμ.

[b] Klason lignin isolated during chromatographic investigation of acid-soluble lignin.

The relatively low methoxyl content of the Klason lignin from BNL (20.3%) may be due primarily to an incomplete removal of p-hydroxybenzoic acid during the Klason lignin determination. Only small differences exist between the methoxyl content of the Klason lignins from wood (21.6-21.8%) and from MWL-A (21.3%), but Björkman (34) also found that MWL gave Klason lignins which were slightly lower in methoxyl than the Klason lignin from wood. This could be relevant to the suggestion of Grohn and co-workers (39) that methoxyl may be split from lignin during the vibratory grinding of wood in the presence of oxygen. On the other hand, Björkman (27) milled wood in both air and nitrogen atmospheres, and found that the differences between air-milled and nitrogen-milled MWL were small. Further consideration of this question may be important in future investigations of the ball milling of wood.

A portion of the 95% ethanol extract of a Klason lignin from extractive-free aspenwood (cf. p. 57) was evaporated to dryness with a stream of air and dried in a vacuum oven at 60°C. The ultraviolet absorption curve of the lignin obtained in this way was determined in aqueous methyl cellosolve on the DK-2 spectrophotometer, and the absorptivity at the far ultraviolet absorption maximum was 99 l./g.-cm. The ultraviolet absorption curves of the alcohol extract of the Klason lignin and of the Klason filtrates from the other lignin preparations are compared in Fig. 13.

An examination of Fig. 13 shows a great similarity between the absorption curve of the Klason filtrate from MWL-A and that from the Klason lignin, and it is surprising that a Klason lignin would yield an acid-soluble lignin which appears to be similar to the acid-soluble lignin from MWL-A. Even that portion of a Klason lignin soluble in 95% ethanol has an absorption curve more characteristic of an unaltered lignin than the acid-soluble lignin from MWL-A. The curve for the acid-soluble lignin from BNL is unlike the other curves, but this can be explained by a liberation of a large amount of p-hydroxybenzoic acid which has an absorption maximum about 251 mμ (90a).

The ultraviolet absorption curves for the Klason filtrate from MWL-A and the ether-extracted Klason filtrate from 1 g. of extractive-free aspenwood (Fig. 6-B) are compared in Fig. 14. The MWL-A curve corresponds to a sample size giving the same amount of Klason lignin (181.3 mg.) as a 1-g. sample of ovendry wood. It appears that MWL-A

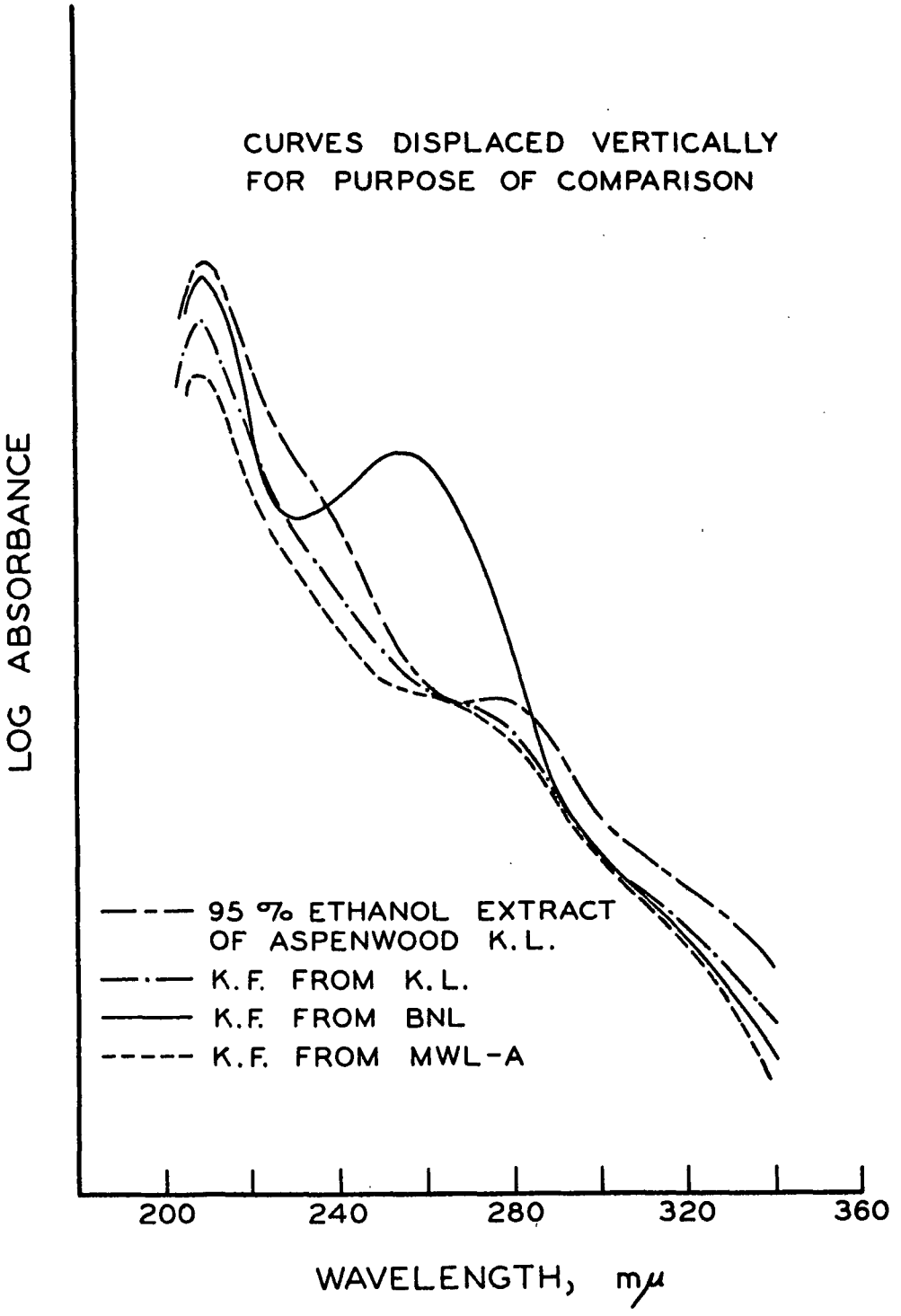

Figure 13. Ultraviolet Absorption Characteristics of Aspen Lignin Preparation

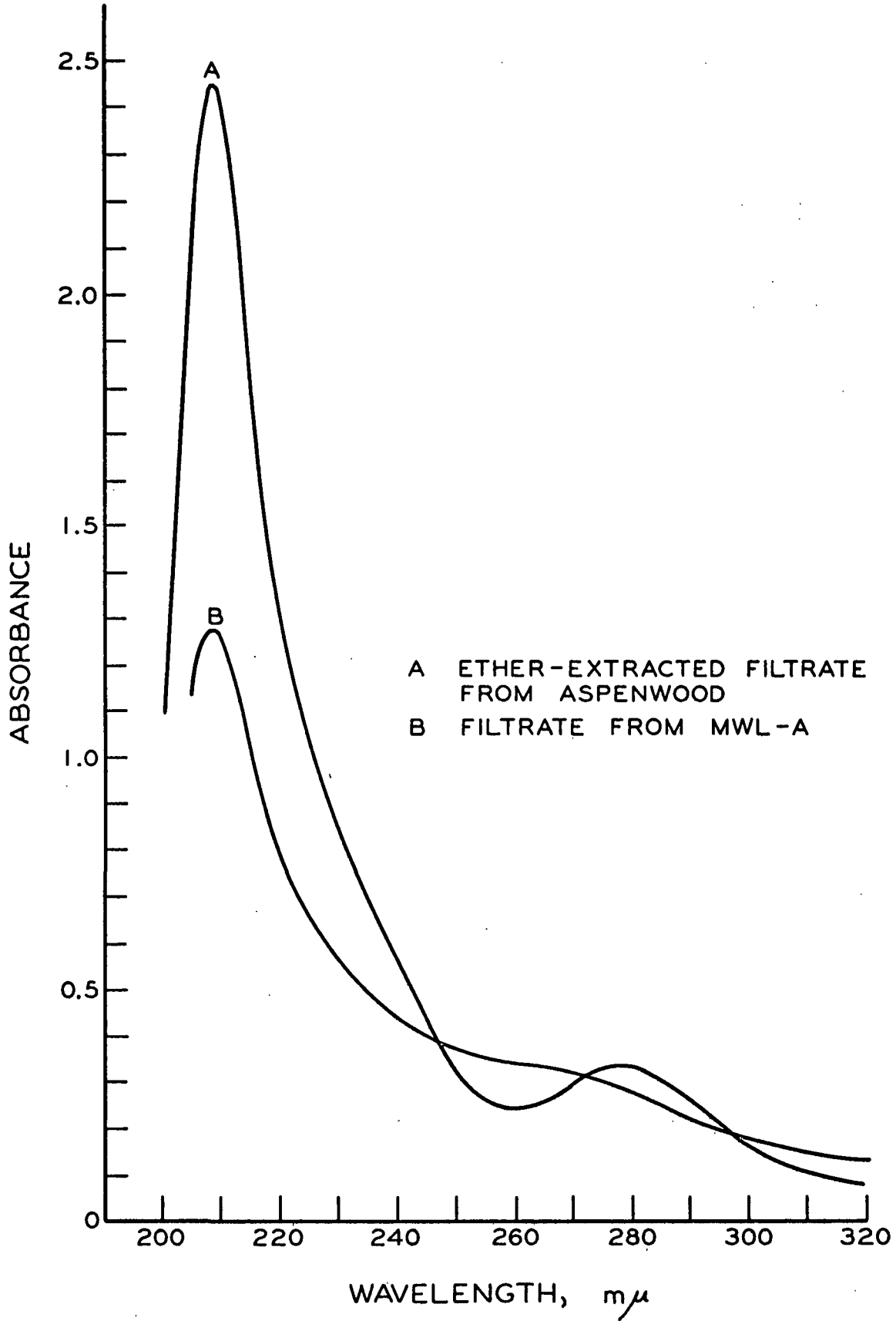

Figure 14. Ultraviolet Absorption Characteristics of the Klason Filtrates from Aspenwood and MWL-A

is quite unlike the protolignin in its reaction to sulfuric acid during the Klason lignin determination. The rather drastic flattening of the MWL-A absorption curve probably indicates that the acid-soluble lignin from MWL-A was altered to a greater extent than the acid-soluble lignin from the wood.

The lignin preparations are compared again in Table XX. A low value for the ratio of the absorbances at the two absorption maxima (ca. 208 and 275 mμ) indicates flattening of the absorption spectrum and probable modification of the lignin. The data are in agreement with the preceding discussion.

The fact that the acid-soluble lignin from MWL-A appears to be altered to a greater extent than the acid-soluble lignin from the wood may indicate that MWL-A is not representative of the total lignin, or that it suffered a general chemical attack in the vibratory ball mill. It may be that the cell wall offers a physical protection which limits the modification of the acid-soluble lignin from the protolignin. It is also possible that the ball mill may have liberated functional groups which were protected by chemical bonds in the wood and which were responsible for condensation of the acid-soluble lignin from MWL-A.

A rupture of chemical bonds between lignin and carbohydrate during the vibratory grinding of wood may be indicated by Björkman's (27, 34, 35) observation that MWL with the least carbohydrate contains more substituted p-hydroxybenzyl alcohol groups per phenylpropane unit than lignin preparations with more carbohydrate such as LCC [Björkman's (35) lignin-carbohydrate complexes] or the wood itself. Recent electrophoretic studies

TABLE XX

ULTRAVIOLET ABSORPTION CHARACTERISTICS OF

ASPENWOOD LIGNIN PREPARATIONS

Sample	$\underline{A}^a/A_{275}$	$\underline{k}^a$
K.L. filtrate from wood after ether extraction	7.4	---
NSSC lignosulfonate	7.3	---
MWL-A in aqueous Methyl Cellosolve	7.0	---
MWL-A in Methyl Cellosolve	7.0	108
MWL(RT) in Methyl Cellosolve	6.7	---
MWL-B in Methyl Cellosolve	6.7	---
BNL in Methyl Cellosolve	6.4	107
BNL in aqueous Methyl Cellosolve	6.1	106
95% Ethanol extract of K. L. in aqueous Methyl Cellosolve	5.9	99
K.L. filtrate from K.L.	4.9	---
K.L. filtrate from MWL-A	4.2	---
K.L. filtrate from BNL	3.6	---

[a] $\underline{A}$ and $\underline{k}$ are the absorbance and absorptivity, respectively, at the far ultraviolet absorption maximum.

(98) on lignin preparations which were isolated from milled wood indicated that lignin is combined chemically with the hemicellulose, and linkages involving the oxygen bridge of beta-phenyl glucosides or of benzyl ethers are presently the most favored possibilities (99). Freudenberg and Grion (100) showed recently, for example, that dehydrogenation of coniferyl alcohol in the presence of cane sugar produced a benzyl ether linkage

between "lignin" and the carbohydrate. Thus, it seems possible that the larger number of substituted p-hydroxybenzyl alcohol groups in MWL may be due to cleavage of benzyl ether linkages between lignin and carbohydrate in the vibratory ball mill.

The condensation of lignin in the presence of acid (Fig. 15) is believed to involve the condensation of reactive groups which are at least partly of benzyl alcoholic character (I) (96). During this condensation, the α-carbon atoms of some of the elements become attached to two aromatic nuclei (II) with the loss of water. Condensation in the 6-position may also result through acid treatment (101).

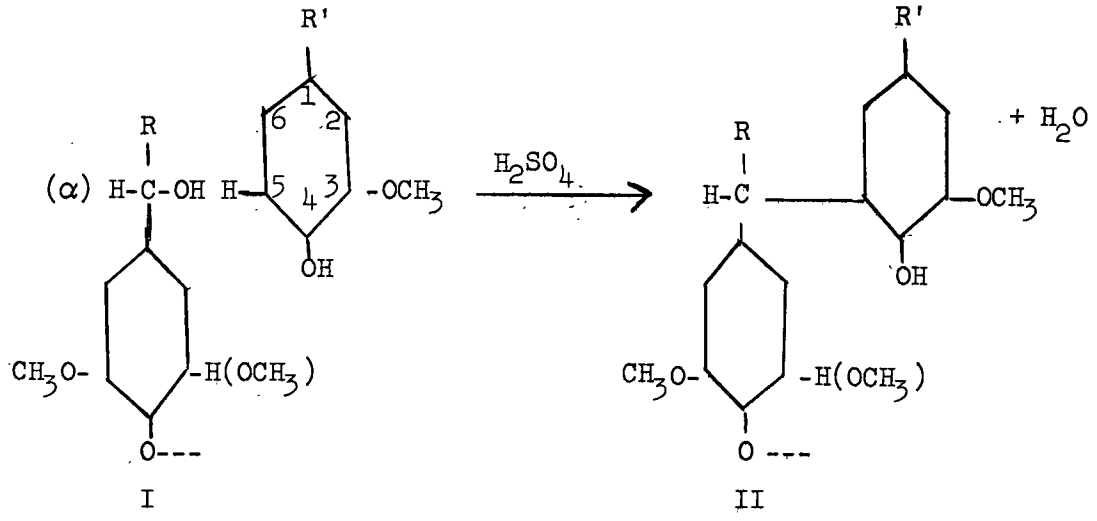

Figure 15. Condensation of Lignin During the Klason Lignin Determination

The differences between the absorption spectra of the acid-soluble lignins from protolignin and MWL-A might only reflect a greater tendency for the acid-soluble lignin from MWL-A to be condensed. Inasmuch as benzyl alcohol groups may be involved in the condensation of lignin, the enhanced condensability of the acid-soluble lignin from MWL-A may be due to benzyl alcohol groups which result from the cleavage of lignin-carbohydrate bonds in the vibratory ball mill. Thus, it seems possible that bonds between lignin and carbohydrate may provide some protection against the condensation of acid-soluble lignin during the Klason lignin determination.

THE QUANTITATIVE DETERMINATION OF LIGNIN

THE DETERMINATION OF ACID-SOLUBLE LIGNIN

Absorptivity of Acid-Soluble Lignin

It was concluded before that MWL-A was a suitable ultraviolet standard for at least 80% of the acid-soluble lignin in the aspenwood Klason filtrate. With the exception of the phenolic acid fraction, MWL-A may also be a suitable standard for the rest of the acid-soluble lignin. The relatively low absorptivity (ca. 50 l./g.-cm. at 208 mμ) of p-hydroxybenzoic acid, the major component of the phenolic acid fraction, lowers the average absorptivity of the acid-soluble lignin. In preference to the absorptivity of MWL-A (108 l./g.-cm.), therefore, an absorptivity of 105 l./g.-cm. was used to calculate the acid-soluble lignin from absorption measurements at 208 mμ. This compensated for the amount of p-hydroxybenzoic acid found previously (Table XVII) in the Klason filtrate.

Carbohydrate Interference at 208 mμ

The effect of carbohydrate hydrolysis products on the ultraviolet absorption at 208 mμ was estimated from the absorption curves presented in Fig. 11; the pertinent data are summarized in Table XXI. The absorptivities used for acid-soluble lignin were 105 and 15 l./g.-cm. at 208 and 280 mμ, respectively. The absorptivity at 280 mμ was calculated from the absorption data for MWL-A presented in the APPENDIX.

TABLE XXI

ULTRAVIOLET ABSORBANCE DATA FROM FIGURE 11

	Absorbance	
	208 mμ	280 mμ
Carbohydrate (estimated)	0.24	2.28
Klason filtrate	5.29	1.80

It was assumed at first that the absorption due to the carbohydrate at 208 mμ was negligible, and the concentration of acid-soluble lignin ($\underline{C}_L$) in the Klason filtrate was estimated from the Beer's law relationship:

$$\underline{C}_L = 5.29/105 = 0.0504 \text{ g./l.} \qquad (2)$$

Therefore, the absorbance of the lignin ($\underline{A}_L$) at 280 mμ was:

$$\underline{A}_L \text{ (280 mμ)} = (15)(0.0504) = 0.76 \qquad (3)$$

and the actual absorbance of the carbohydrate hydrolysis products ($\underline{A}_C$) at 280 mμ was estimated by difference:

$$\underline{A}_C \text{ (280 mμ)} = 1.80 - 0.76 = 1.04 \qquad (4)$$

It was assumed that the ratio of the actual carbohydrate absorbance to the estimated carbohydrate absorbance was the same at 208 mμ as it was at

280 mμ in order to calculate the actual carbohydrate absorbance ($\underline{A}_C$) at 208 mμ:

$$\underline{A}_C \ (208 \text{ mμ}) = (1.04/2.28)(0.24) = 0.11 \qquad (5)$$

and the percentage of the total absorption at 208 mμ due to carbohydrate was estimated:

$$(0.11/5.29)(100) = 2.1\% \qquad (6)$$

Equation (6) showed that when the Klason filtrate is refluxed, the carbohydrate hydrolysis products represent only 2% of the total light absorption at 208 mμ. This confirmed the original assumption, and the calculations were found to be valid.

Marth (47), however, studied the absorbance of the aspenwood Klason filtrate at 280 mμ. He found that the absorbance was doubled when the filtrate was boiled with reflux rather than without, and this indicated that the carbohydrate interference is also twice as great when the filtrate is refluxed. Thus, only 1% of the total light absorption at 208 mμ should be attributed to the carbohydrate hydrolysis products when the filtrate is not refluxed.

The Components of Ultraviolet Absorption at 208 mμ

The absorbance of the Klason filtrate (1000 ml.) from 1 g. of extractive-free aspenwood was approximately 3.0 absorbance units at 208 mμ, but ether extraction removed absorbing substances equivalent to 20% of the total absorption. At a concentration of 2 mg./l., p-hydroxybenzoic

acid contributes 0.1 absorbance units, and the contribution of the phenolic acid fraction or "lignin acids" is estimated at less than 0.15 absorbance units. With the assumption that carbohydrate hydrolysis products are responsible for 1% of the total absorption, the breakdown presented in Table XXII was compiled.

TABLE XXII

COMPONENTS OF ULTRAVIOLET ABSORPTION OF ASPENWOOD KLASON FILTRATE

	Absorption at 208 mμ, %
Carbohydrate hydrolysis products	1
Lignin acids	5
Ether-soluble lignin	14
Nonextractable lignin	80
	100

Calculation of Acid-Soluble Lignin

The Klason filtrate[1] from a 1.000-g. sample of extractive-free aspenwood had an absorbance of 3.04 absorbance units at 208 mμ when it was diluted to 1000 ml. The filtrate was boiled without reflux, and the carbohydrate interference was neglected in calculating the concentration (C_L) of the acid-soluble lignin:

[1] Klason filtrate used in previous study of Beer's law relationship.

$$\underline{C}_L = 3.04/105 = 0.029 \text{ g./l.} \qquad (7)$$

Inasmuch as the volume of the filtrate was also 1 liter, Equation (7) showed that 29 mg. of acid-soluble lignin were formed from the 1-g. sample of wood.

SECONDARY ERRORS IN THE KLASON LIGNIN DETERMINATION

The carbohydrate hydrolysis products condense with the Klason lignin thereby increasing the "lignin content" of the wood. The importance of this effect was estimated from the absorbance ($\underline{A}_F$) of the hydrolysis products which combined with the lignin. $\underline{A}_F$ was calculated as the difference between the estimated (Table XXI) and actual [Equation (4)] absorbance of the carbohydrate hydrolysis products at 280 mμ:

$$\underline{A}_F \text{ (280 m}\mu\text{)} = 2.28 - 1.04 = 1.24 \qquad (8)$$

The major component condensed with the lignin was assumed to be furfural with an absorptivity of 150 l./g.-cm. at 280 mμ, and the equivalent concentration ($\underline{C}_F$) of the condensed furfural was calculated:

$$\underline{C}_F = 1.24/150 = 0.0083 \text{ g./l.} \qquad (9)$$

Because the volume of the filtrate was only 575 ml., the condensed furfural was:

$$(0.0083)(0.575) = 0.0048 \text{ g.} \qquad (10)$$

Equation (10) indicates that 4-5 mg. of carbohydrate hydrolysis products may condense with the lignin when the Klason filtrate is refluxed.

It was observed that the Klason lignin values were equivalent to 18.4% of the wood if the filtrate was refluxed, and only 18.1-18.2% if the filtrate was not refluxed. Presumably, more furfural condensed with the lignin under reflux when it was not allowed to steam distill. It follows that the interference is probably less, possibly only 1-2 mg., when the Klason filtrate is not refluxed.

Other factors tending to increase or decrease the Klason lignin are also of interest. Marth (47) found that the sulfur content of the aspenwood Klason lignin was generally 0.2% or more, and this was attributed mainly to sorption of sulfuric acid. On the other hand, formaldehyde or methoxyl may be split off, and water may be lost due to condensation of the lignin. Thus, the secondary errors tend to cancel each other, and it is possible that the value for the Klason lignin (discounting loss of acid-soluble lignin) may be essentially correct.

THE TOTAL LIGNIN CONTENT

The total lignin content of the extractive-free aspenwood was estimated by adding the weight of the acid-soluble lignin to the weight of the Klason lignin. The value obtained indicated that the wood contained about 21.0% lignin, whereas the Klason lignin itself represented only 18.1% of the wood. Thus, it appeared that approximately 15% of the lignin was solubilized during the Klason lignin determination.

The data for the Klason lignin determination on the wood and other lignin preparations (Table XIX) are compared in Table XXIII. All results

TABLE XXIII

KLASON LIGNIN DETERMINATION ON WOOD AND LIGNIN PREPARATIONS[c]

Sample	Weight, mg.	Lignin, %	Lignin, mg.	K.L., mg.	Methoxyl in K.L., %	Acid-Soluble[a] Lignin, mg.	Absorbance of[b] Filtrate
MWL-A	227.3	91	206.8	181.3	21.3	25.5	1.279
MWL-A	226.7	91	206.3	181.3	21.3	25.0	1.252
BNL	204.1	100	204.1	181.3	20.3	22.8	0.952
K.L.	191.8	100	191.8	181.3	--	10.5	0.451
Wood	1000.0	21.0	210.3	181.3	21.8	29.0	3.04

[a] The acid-soluble lignin (except for the wood) was calculated as the difference between the lignin in the original preparation and the resultant Klason lignin.

[b] Absorbance at 208 mμ and 1000 ml.

[c] Data calculated from Table XIX on basis of 181.3 mg. Klason lignin.

were recalculated to correspond to a starting sample giving the same amount of Klason lignin (181.3 mg.) as a 1-g. sample of wood. The wood gave the most acid-soluble lignin whereas the more condensed Klason lignin gave the least. A calculation based on the methoxyl content in MWL-A and its Klason lignin indicated the formation of 24.5 mg. of acid-soluble lignin from a 227-mg. sample of MWL-A. This agrees remarkably well with the values in Table XXIII.

The conclusions regarding the formation of acid-soluble lignin and the total lignin content of the wood are applicable only to Populus tremuloides, and more specifically to the sample used in this investigation. The same approach may be useful in studying the lignin content of other hardwood species, but even this has yet to be demonstrated.

SUMMARY

The present investigation attempts to resolve the uncertainty regarding the status of the Klason lignin determination as applied to aspenwood (Populus tremuloides).

A vibratory ball mill was constructed which allowed sub-zero grinding temperatures to be maintained, and extractive-free aspenwood was milled for 25 hr. at -78°C. in the preparation of milled wood lignin. The milled wood was extracted with aqueous dioxane, and the lignin isolated from the extract in accordance with Björkman's (34) "standard procedure" was designated MWL-A. MWL-A was obtained in a yield of 13.0% based on the Klason lignin. It contained approximately 91% lignin and 9% carbohydrate, and xylan was the major component of the carbohydrate fraction.

When extractive-free aspenwood was subjected to the Klason lignin determination, about 76% of the methoxyl was found in the Klason lignin. Only 8% of the methoxyl was split off, and the relatively high methoxyl content (16%) of the Klason filtrate indicated the solubilization of lignin during the standard Klason procedure.

A chromatographic examination of the Klason filtrate revealed about 2 mg. of p-hydroxybenzoic acid and lesser amounts of syringic and vanillic acids for each gram of extractive-free wood. Apparently, these "lignin acids" are cleaved from the lignin during the Klason lignin determination. The Klason lignin itself contains about 2% combined p-hydroxybenzoic acid, and it appears that p-hydroxybenzoic acid is a significant part of both the acid-soluble lignin and the Klason lignin.

The spectrophotometric studies showed that the far ultraviolet absorption maximum may be used to determine acid-soluble lignin in the aspenwood Klason filtrate with very little (ca. 1%) interference from the carbohydrate hydrolysis products. Although there was some variation in the position of the absorption maximum (208-209 mμ) over the usable concentration range, the Beer's law relationship was valid at 208 mμ.

The spectrophotometric studies also demonstrated that the acid-soluble lignin was formed during the initial treatment with 72% sulfuric acid, and not during the secondary hydrolysis with dilute acid. Approximately 20% of the acid-soluble lignin could be extracted with ether, but "lignin acids" accounted for only 25% of the ether-soluble fraction.

The acid-soluble lignin which was not extracted from the Klason filtrate with ether (ca. 80%) has an ultraviolet absorption curve which is steep, sharp, and distinct, and remarkably similar to the absorption curves of MWL-A and of an aspen lignosulfonate. This indicates that the acid-soluble lignin was not severely altered during the Klason lignin determination, and apparently MWL-A can be considered a satisfactory ultraviolet reference standard for most of the acid-soluble lignin in the aspenwood Klason filtrate.

MWL-A gave almost as much acid-soluble lignin as the protolignin when subjected to the Klason lignin determination, but the rather drastic flattening of the ultraviolet absorption curve of the Klason filtrate suggest that the acid-soluble lignin from MWL-A was altered to a greater extent than the acid-soluble lignin from the wood. This may only reflect

an enhanced condensability of the acid-soluble lignin from MWL-A due to benzyl alcohol groups which are formed as a result of the cleavage of lignin-carbohydrate bonds in the vibratory ball mill. Thus, it seems possible that bonds between lignin and carbohydrate may provide some protection against the condensation of acid-soluble lignin during the Klason lignin determination.

The total lignin content of the wood was estimated as the combined weight of the acid-soluble lignin and the Klason lignin. MWL-A was used as a standard in calculating the acid-soluble lignin from ultraviolet absorption measurements at 208 mμ, and the calculation indicated the formation of 29 mg. of acid-soluble lignin from each gram of wood. Inasmuch as the Klason lignin represented 18.1% of the wood, the total lignin content was estimated to be 21.0% of the wood. It appears that approximately 15% of the lignin was solubilized during the Klason lignin determination.

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APPENDIX I

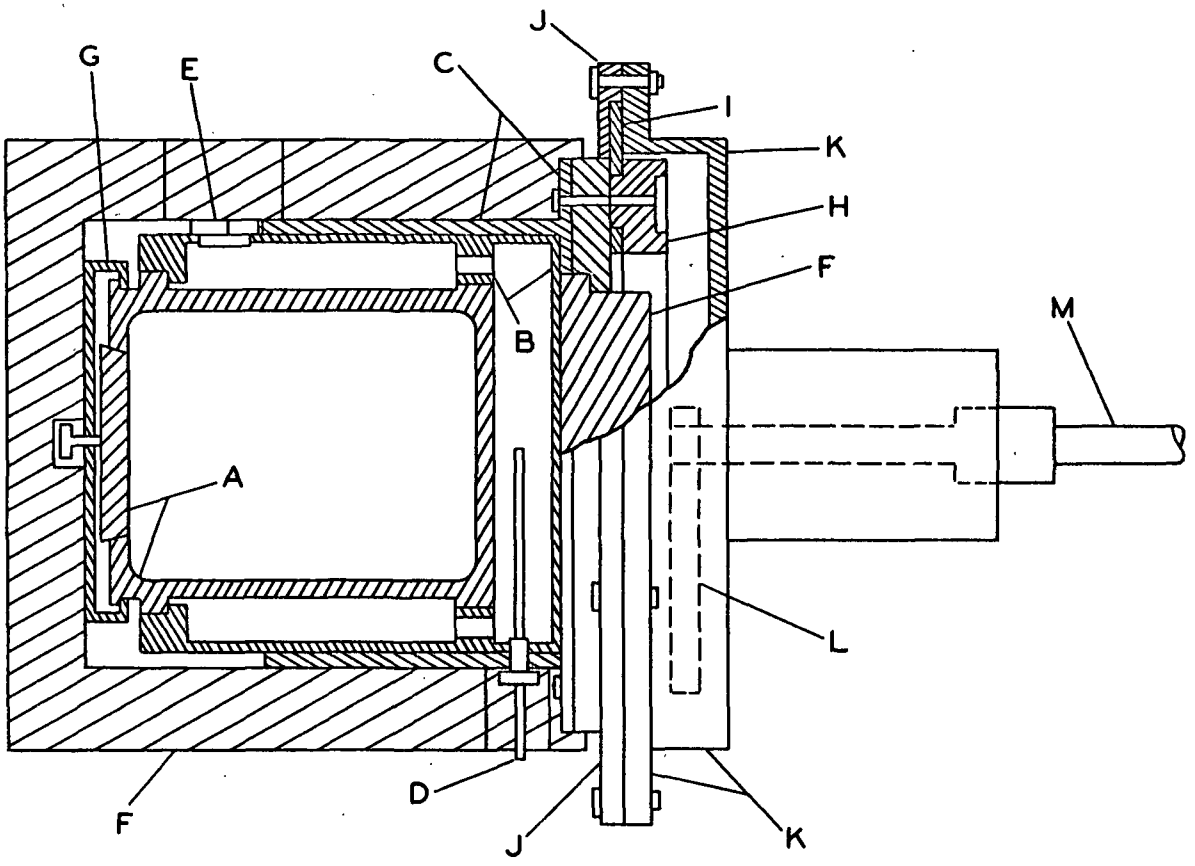
THE VIBRATORY BALL MILL

The following discussion is confined primarily to the peculiarities in the design and operation of the vibratory ball mill employed in this investigation. A schematic drawing of the jar assembly is presented in Fig. 16, and further details can be found in the working drawings[1]. Those features of the mill not shown in Fig. 16 reflect the basic design of the N.B.S. mill.

The outer surface of the cylindrical jar, excluding the cover face, is surrounded by a concentric cooling chamber. The cooling chamber supports the jar in the back by means of a close-fitting ring or jar support, perforated for continuity between the side and back sections of the cooling chamber. The jar screws into the front of the cooling chamber, and abuts against a projection on the inside of the chamber. A thin teflon gasket (not shown) assures an airtight seal between the jar and the cooling chamber.

The jar and cooling chamber fit into a holder which is fastened in turn to a slip ring. The slip ring is insulated from the holder in order to prevent heat conduction from the housing to the cooling chamber. The cooling chamber, secured only with two bolts (not shown) to the holder, can be slipped in and out of the holder quite easily in the course of the normal operation of the mill. The jar, cooling chamber, holder, and the

[1] A complete set of detailed working drawings is on file at the Plant Engineer's Office.



A	JAR & JAR COVER
B	JAR COOLING CHAMBER WITH JAR SUPPORT
C	JAR HOLDER
D	GAS OUTLET TUBE
E	REMOVABLE FILLING PLUG
F	STYROFOAM INSULATION
G	JAR COVER CLAMP
H	MICARTA INSULATION
I	SLIP RING
J	SLIP RING CLAMP
K	EXCENTER & BEARING HOUSING
L	EXCENTER
M	SHAFT

Figure 16. Jar Assembly for Vibratory Ball Mill

slip ring rotate automatically as a unit due to the unusual vibratory action of the mill, and the rate of rotation (ca. 1 r.p.m.) is governed by the clearance between the slip ring and housing.

When grinding at sub-zero temperatures, the entire jar assembly is covered with a thick layer of styrofoam. A separate piece is placed in the back of the cooling chamber also to reduce the heat transfer from the bearings and housing by convection and radiation. The coolant is added directly to the cooling chamber after the filling plug is removed, and the evaporated coolant escapes through the gas outlet. The gas outlet extends to the axis of rotation, and the cooling chamber can be filled approximately one-half full without any appreciable loss of coolant, even though it makes a complete revolution. If the mill is allowed to run when adding solid carbon dioxide (finely pulverized with a hammer), and the rotation is stopped by restraining the jar or inserting a pin through the slip ring and housing, the carbon dioxide will quickly distribute itself through the cooling chamber. Nearly the entire chamber can be filled in this way, and only a small part of the carbon dioxide seems to be lost before it is evaporated.

Various coolants were tried, but carbon dioxide (-78°C.) turned out to be most generally satisfactory. Liquid nitrogen (-196°C.) could not be used effectively or economically, because the jar was not insulated well enough to sustain the large temperature gradient. The use of a liquid nitrogen-95% ethanol slurry (-115 to -125°C.) was feasible, but not practical for long grinding periods; it was necessary to add liquid

nitrogen every fifteen minutes. The 95% ethanol-carbon dioxide slurry (-72°C.) can be used quite easily, but the styrofoam insulation is not completely resistant to alcohol. The alcohol was added originally to improve the heat transfer coefficient between the jar and coolant, but the vibratory action of the mill itself greatly reduces the normal air-film resistance to heat transfer. This can be observed when the jar is cooled from room temperature to -78°C.; the gas evolution from the cooling chamber is much greater when the mill is in operation than when it is stopped. Therefore, it is unlikely that the actual jar temperature will be lower with alcohol-carbon dioxide (-72°C.) than with carbon dioxide alone (-78°C.) in the cooling chamber, and carbon dioxide appeared to be the most practical coolant for routine grinding. The demand for coolant drops off very rapidly as the jar temperature is reduced, and it is only necessary to add carbon dioxide every one to two hours to maintain a detectable evolution of gas from the chamber.

APPENDIX II

THE URONIC ACID DETERMINATION

Uronic acids and complex polyuronides can be decarboxylated upon boiling with 12% hydrochloric acid to yield the theoretical amount of carbon dioxide. This was the basis for the quantitative determination of uronic acids associated with the lignin preparations.

The apparatus described by Browning (102) for the macrodetermination of polyuronide carboxyl was scaled down, without any changes in the basic design, and used for a microdetermination in this investigation.

At the start of each run, 85% phosphoric acid-silver phosphate solution was added to the trap, and cooling water started through the reflux condenser. The weighed sample and 17-20 ml. of 12% hydrochloric acid saturated with sodium chloride were added to the cold reaction flask just before it was attached to the apparatus. The carbon dioxide absorption tube was placed in position, and the apparatus was flushed with 9-10 ml. of nitrogen per minute for 1 hr. at room temperature.

When flushing was completed, the absorption tube was removed from the apparatus without disturbing the flow of nitrogen. It was weighed on a microbalance, and replaced on the apparatus. The nitrogen flow rate was reduced to approximately 5 ml. per minute, and the glycerine heating bath was positioned under the reaction flask, being careful to keep the glycerine level below the level of the liquid in the flask. Heating was started to increase the glycerine bath temperature slowly to 134-137°C. in about 1 hr.

After 3 hr. at 135°C., the absorption tube was removed and weighed again. This required about ten minutes, during which time the apparatus continued to run without being disturbed. The absorption tube was replaced on the apparatus while the reaction was continued for an additional 1-1/2 hr. The incremental weight increase during this 1-1/2-hr. period was doubled and subtracted from the initial 3-hr. increase to compensate for nonuronic acid carbon dioxide evolution.

A certain amount of foaming was noted with these lignin materials, and a silicone defoamer was used in one analysis. A blank determination indicated that it might give a small amount (63 micrograms) of carbon dioxide itself.

APPENDIX III

THE XYLOSE AND GLUCOSE EQUIVALENT OF ASPENWOOD CARBOHYDRATE

The xylose and glucose equivalent of aspenwood carbohydrate was calculated for the purpose of estimating the effect of the carbohydrate on the ultraviolet absorption characteristics of the aspenwood Klason filtrate. The calculation was based on the summative analysis shown in Table XXIV, which was compiled from the acetyl analysis reported by Thomas (18) and the wood analysis reported by Quick (86). The pentosans were considered to be xylan, and the hexosans including a small percentage (0.5%) of rhamnan were considered to be glucan.

TABLE XXIV

SUMMATIVE ANALYSIS OF ASPENWOOD	
Alcohol-benzene extract	4.3
Lignin	21.2
Uronic anhydride	5.0
Pentosans (xylan)	18.7
Hexosans (glucan)	45.2
Acetyl	3.4
	97.8%

The polyuronides are known to form furfural and undergo decarboxylation when treated with acid (103), and they were grouped with the xylan for the purpose of this calculation. Correcting uronic anhydride to its xylan equivalent by the appropriate weight ratio:

$$(5.0)(132/176) = 3.8\% \text{ xylan} \quad (11)$$

it was found that the holocellulose contains 45.2% glucan and

$$18.7 + 3.8 = 22.5\% \text{ xylan} \quad (12)$$

This was corrected to an extractive-free basis:

$$22.5/0.957 = 23.5\% \text{ xylan} \quad (13)$$

$$45.2/0.957 = 47.2\% \text{ glucan} \quad (14)$$

Therefore, the sugar equivalent of 1 g. of extractive-free aspenwood was estimated:

$$(0.235)(150/132) = 0.267 \text{ g. xylose} \quad (15)$$

$$(0.472)(180/162) = 0.524 \text{ g. glucose} \quad (16)$$

$$0.791 \text{ g. total sugar}$$

APPENDIX IV

TABLE XXV

ULTRAVIOLET ABSORPTION OF LIGNIN PREPARATIONS IN FRESHLY DISTILLED METHYL CELLOSOLVE

	Absorbance			
λ, mμ	BNL 5.67 mg./l.	MWL-A 7.16 mg./l.	MWL-B 12.64 mg./l.	MWL(RT) 7.37 mg./l.
340	0.011	0.004	0.009	0.009
330	0.017	0.011	0.018	0.015
321	0.020	0.021	0.027	0.022
310	0.025	0.030	0.038	0.033
300	0.033	0.039	0.050	0.045
290	0.048	0.062	0.072	0.069
280	0.082	0.095	0.097	0.100
275	0.095	0.101	0.100	0.110
270	0.100	0.100	0.095	0.109
260	0.110	0.097	0.085	0.100
250	0.130	0.125	0.108	0.129
240	0.195	0.222	0.195	0.222
230	0.229	0.285	0.259	0.285
225	0.251	0.322	0.300	0.322
220	0.340	0.420	0.380	0.425
218	0.414	0.499	0.455	0.512
216	0.500	0.598	0.550	0.618
215	0.543	0.642	0.595	0.664
214	0.574	0.678	0.632	0.708
213	0.601	0.702	0.662	0.735
212.5	0.607	0.704	0.669	0.742
212	0.605	0.696	0.667	0.738
211	0.586	0.658	0.644	0.708
210	0.529	0.570	0.572	0.618
209	0.405	0.430	0.438	0.472